"THE GROWTH, METASTASES AND HORMONAL SENSITIVITIES OF HUMAN MELANOMAS IN THE NUDE MOUSE"

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TO MY DEAR

PARENTS

ABSTRACT

This thesis records the results of a series of experiments on the kinetics of growth of 7 human malignant melanomas in nude mice, with particular reference to factors in the host "milieu" that modulate proliferation, metastasis and phenotypic expression of the tumours.

Melanoma cell lines were established *in vitro*, from biopsy material obtained from 7 patients with metastatic malignant melanoma. Two of these lines synthesized tyrosinase and melanin at a rate that was directly related to cell density. The five remaining lines did not pigment *in vitro*. With the exception of one line they all gave rise to tumours when inoculated subcutaneously into nude mice. All 7 lines were aneuploid and 5 of the lines showed anchorage-independent growth *in vitro*.

The growth rate and latency period of these human melanoma cell lines has been studied in nude mice as a function of inoculum size. Considerable variation was observed and growth rate *in vivo* correlated poorly with the doubling times of the corresponding cells cultured *in vitro*.

Passage through nude mice had no effect on *in vitro* phenotypic characteristics of cell lines. In spite of prolonged *in vitro* passage, in a number of instances the histological appearances of the mice tumours were very similar to those seen in the original patient.

Coinjection of either adult or juvenile skin fibroblasts with subtumorigenic doses of melanoma cells promoted the growth of tumours that would otherwise not have formed.

One of the melanoma cell lines grew rapidly in a male host at an inoculum level that did not give rise to tumours in the female mouse, showing that hormonal influences in the host have a definitive effect on the growth of some

melanomas *in vivo*. Castrated male mice also failed to support the growth of this melanoma cell line. Addition of estrogen and dihydrotestosterone pellets to the castrated and ovariectomised mice proved in certain cases to be effective stimulants of tumour growth.

By excising the primary tumour before it reached a size which was lethal to the host, metastases were made evident in a number of instances. Two of the six melanoma cell lines showed metastatic melanoma deposits in 100% of the animals inoculated. This occurred within 12 weeks of removal of the primary tumour. In one of the two cell lines metastatic spread was directly related to the size of the primary tumour.

One of the cells lines, UCT-Mel 7 elicited an intense desmoplastic response in the host and the tumours were heavily infiltrated with host macrophages and fibroblasts. This cell line had a most unusual behaviour pattern in the mouse as it grew exponentially for a fairly brief period after which a plateaux phase occurred during which no growth took place. This was followed by a phase of regression and then a prolonged period of dormancy after which a rapid exponential growth phase followed. Tumours transplanted to new mice during this latter phase gave rise to rapidly growing neoplasms which were lethal to their hosts.

CONTENTS

ABBREVIATIONS

Cnt	-	Control
Cx	-	Castration
DB	-	Dulbecco's modified Eagle's medium
DHT	-	5-dihydrotestosterone
DMSO	-	Dimethyl sulphoxide
E2	-	17β-estradiol
EDTA	-	Ethylene-diamine-tetra acetate
ETOH	-	Ethanol alcohol
F	-	Female
FCS	-	Foetal calf serum
FC10	-	10% FCS
Hr	-	Hour(s)
i.p.	-	Intraperitoneal
LDH	-	Lactate dehydrogenase
M	-	Male
Min	-	Minute(s)
PBS	-	Phosphate buffered saline (0.14M NaCl; 2.7mM KCl 8mM Na_2HPO_4 ; 1.5 mM KH_2PO_4)
Prog	-	Progesterone
RPMI	-	Roswell Park Memorial Institute culture medium 1640
RPMI-10	-	RPMI medium with 10% FCS
s.c.	-	Subcutaneously
Sx	-	Sham-operated
TAM	-	Tamoxifen
TBS	-	Tris-buffered saline (0.14M NaCl; 5mM KCl; 0.7mM $NaHPO_4$ 24.8mM Tris.HCl, pH 7.4)
TD50	-	Number of cells required for 50% take

Td - Doubling time

T(del) - Delay time

TD - Tris-Dulbecco's saline

0.14M NaCl; 5mM KCl; 0.7 mM Na_2HPO_4

24.8 mM Tris.HCl, pH 7.4.

INTRODUCTION

INTRODUCTION

Since Laennec first described malignant melanoma in 1812 (Laennec, 1812), the disease has increasingly attracted the attention of clinicians, pathologists, epidemiologists and biologists. The reasons for this interest are not difficult to define.

In the first instance, malignant melanoma is an aggressive disease with a particular tendency to affect fair-skinned, first world populations with a tumour that is usually relentlessly progressive and incurable. Moreover, the incidence of the disorder is increasing at an annual rate of about 6% (Elwood and Lee, 1974; Elwood et al., 1975).

Before 1950 malignant melanoma was classified with other skin cancers so that early epidemiological data are somewhat sketchy. Scandinavian and Australian records covering the past 35 years have, however, been meticulously collected and these show an increasing incidence rate with a doubling time of about 10 years. Current figures for Scandinavia are approximately 12 new cases per 100 000 population per year (Mackie and Young, 1984) and those for Australia approximately 32 new cases per 100 000 population per year.

A large amount of descriptive data has been assembled in an attempt to relate this increase to possible predisposing causes in the host or to initiating or promoting circumstances in the environment. Insulation (Acton et al., 1983), genetic factors (Anderson, 1971; Wallace et al., 1971), hormonal influences (Nesbit, 1979; Rampen and Mulder, 1980; Shaw et al., 1978) cigarette smoking (Shaw and Milton, 1981; Holt and Keast, 1977), contraceptive use (Lerner et al., 1979; Helmrich et al., 1984; Beral et al., 1977; Holly et al., 1983; Lederman et al., 1985) complexion (Beral et al., 1983; Gellin, et al., 1969; Reintgen et al., 1982) and trauma (Allen and Spitz, 1953; Balch et al., 1980; McGovern et al., 1982) are some of the agents that have been considered, but none has emerged as the single most likely etiological

factor. The reasons for the increase remain obscure and a challenge to epidemiologists.

Secondly, therapy of the disease has, on the whole, been disappointing. Early excision of Clark Stage I primary lesions undoubtedly carries a better 5 year survival rate (approximately 79%) than does removal of Stage II lesions where the results are poorer (approximately 34% 5 year survival rate)(Shah and Goldsmith, 1972; Rogers et al., 1983). It is not entirely clear, however, how much this improvement represents the "lead time" effect of earlier diagnosis, the effect of natural selection for less aggressive disease or the real effect of instituting treatment before spread has occurred. Definitive data that confirm, beyond all doubt, the therapeutic advantage of early surgery are lacking. Despite the uncertainties, it is not surprising that continued interest and effort has been directed towards the therapy of an easily diagnosable tumour that starts in a superficial and readily accessible organ and that should, by all accounts, be amenable to early surgery that is curative and non-mutilating.

Thirdly, for the pathologist the disease presents the challenging problem of a neoplasm that originates in a well-defined population of cells, all of which express a very similar, well differentiated phenotype. Furthermore, in the early stages of the disease, most malignant melanomas are histologically very much alike. Despite these similarities the natural history of malignant melanoma is entirely unpredictable. The literature abounds with accounts of striking differences in growth rates, in the period of dormancy after excision, and in metastatic potential and a number of cases have been documented in which spontaneous remission has taken place (Balch et al, 1983; Morton et al.,1974; Cole, 1974). To what then, if the tumour is so similar in other respects, can these differences be attributed and how might the natural history of the disease be predicted from the early histology?

Finally, for the biologist, these neoplasms have been a ready source of

starting material for the development of robust *in vitro* cell lines. Soon after the first development of cell culture techniques, Burrows, (1914) reported his succcessful attempts to grow human malignant melanoma cells *in vitro*. In the 1930's Cloudman (Cloudman, 1941) established a transplantable murine melanoma tumour in DBA 2/J mice. This Cloudman S91 melanoma was subsequently adapted to culture by Yasumura et al. (1966) and in 1973 Pawelek et al., reported that the Cloudman cells responded to melanotropin by increasing tyrosinase activity, melanin formation and changes in morphology.

Since that time numerous descriptions of short-term cultures of animal and human melanoma cells have appeared. Wellings et al. (1960) grew a human malignant melanoma *in vitro* for a period of 4 weeks and Moore et al., in 1962 reported the establishment of several long term human melanoma cell lines. In the last decade numerous authors have reported the successful establishment of human melanoma cell lines from primary or metastatic tumours (Gerner et al., 1975; Liao et al., 1975; Giovanella et al., 1976, Oettgen et al., 1968; McCormick et al., 1983; Tveit et al., 1980; Hoal-van Helden et al., 1986).

These lines have a provided a useful system for examining processes that are relevant both to this particular class of tumours and, at the same time, to cellular biological phenomena of a more general nature.

Melanogenesis, for example, is an obvious and readily quantifiable marker of cellular differentiation that can be used to study factors that govern expression of the differentiated phenotype in pigmenting cell lines. Furthermore, as derivatives of the embryonic neural crest, melanoma cells share a common origin with a variety of ectomesenchymal structures, cells of the autonomic nervous system, calcitonin-producing cells, cells of the carotid body and sensory neurones in spinal ganglia and cranial nerves. It is now clear that, in choosing one of these diverse alternative destinies, pluripotential neural crest "stem-cells" are influenced by environmental cues that come from neighbouring embryonic structures(Le Douarin, 1982). Melanoma

cells thus offer potential for studying cellular interactions that influence morphogenesis or commitment to a particular developmental programme.

As with other neural crest cells, pre-melanocytes are remarkable for their striking ability to migrate to distant target organs where they assume residence and complete their terminal differentiation. These invasive and organotropic attributes have obvious implications for the study of tumour spread and correlate well with the known tendency of malignant melanomas to metastasize in vivo.

Although circumstantial considerations suggest that metastasis requires an aggressive cell type situated in a permissive host environment, the determinants of aggressiveness or permissiveness have not been satisfactorily defined.

Malignant melanomas may spread to intra-epidermal regions (horizontal spread) with formation of satellite lesions. They may penetrate into the depth of the skin (vertical spread) and finally they may gain access to lymphatic channels and blood vessels giving rise to metastasis in lymph nodes or distant organs. In all of these cases it is reasonable to conclude that the metastatic process involves the breaching of such anatomical barriers as endothelial surfaces, inter-cellular junctions, basement membranes or dense fascial planes. One presumes that some form of proteolytic or other destructive function is involved in the traversal that this implies, but the protease or lytic force has not yet been identified with certainty.

It is also well known (Das Gupta and Brasfield, 1964; Meyer and Stolback, 1978; Amer et al., 1979) that malignant melanomas often metastasise to organs that are infrequent targets for metastatic spread from other primary tumours. Furthermore, only a fraction of tumour cells introduced directly into the circulation will settle and develop into secondary tumours (Garcia et al., 1963). These observations have suggested the existence of some form of "adhesive" interaction between melanoma cells and the capillary endothelium in

the target organ or, alternatively, of some sort of local hormonal or metabolic milieu that is propitious for seeding and local survival. Once again, the nature of these influences is obscure.

As far as human tumours go, melanomas are fairly immunogenic, and numerous investigators have documented the existence of melanoma antigens that display various degrees of specificity.(Hersey et al., 1983; Houghton, et al., 1982; Winchester et al., 1978; Hollinshead et al., 1982). Clearly, the development of melanoma-specific antibodies that did not react with epitopes present on other cell types, would hold great promise for the diagnosis and immunotherapy of this class of neoplasms.

My own interest in melanoma research over the past five years has involved the study of tumours that arise when human melanoma cells are inoculated into nude mice. This biological and experimental pathological approach to the study of the disease has, I believe, been useful in the following respects.

Firstly, by using well-defined and homogeneous cell lines, I have been able to examine factors in the host and cellular factors as they interact or as they contribute separately to the behaviour of the tumour <u>in vivo</u>. It transpired that the hormonal milieu into which the tumour cells were inoculated, the size of the inoculum and the co-inoculation of fibroblasts or other cells had a profound effect upon the success with which tumours could be established and upon subsequent rate of growth in the host. It was also apparent that, striking as these effects were, they were dependent, to a greater or lesser extent, on the cell line that was used to produce the tumour.

In the case of one interesting cell line, the interactions between the melanoma cells and the host were complex and seemed, eventually, to lead to the emergence of a mutant tumour cell line that grew much more aggressively than the initial passage from which it was derived. To the best of my know-

ledge this is the first recorded instance of a human tumour cell line that has emerged from a period of prolonged dormancy to assume characteristics that it did not possess originally. It is well known that human tumours may behave in this way under ordinary clinical circumstances and it is indeed encouraging to feel that we may now have a model for studying this phenomenon.

I was also fortunate in that I was able to develop an unusual melanoma cell line that disseminated widely in every nude mouse into which it was inoculated. This consistent tendency for the tumour to spread enabled me to note the effects of hormonal and other manipulations upon the occurrence of metastasis. The most important finding here, perhaps, was the observation that tumours derived from this cell line displayed a critical volume above which metastatic spread occurred with a great frequency and below which metastatic spread occurred only infrequently. Under the controlled conditions of the laboratory, therefore, it has been possible to justify the belief that early surgery, by removing a tumour before a critical mass has been reached, may have beneficial consequences that are independent of the natural history of the tumour.

I have also addressed the question of whether or not melanomas growing in the nude mouse bear any relation to melanomas growing in the original human host. While there are, quite clearly, differences between the two host environments, there are a number of important similarities that could be documented and a number of differences that could be quantitated. All of these, I believe, contribute something to our understanding of this particular class of tumours.

In the chapter that follows immediately upon this introduction, I describe the growth of melanomas in nude mice as I have observed the phenomenon. I give an account of the procedures I have developed for quantitating growth kinetics; for documenting the latency period and for correlating the behaviour <u>in vivo</u> with the behaviour of the parent cell line <u>in vitro</u>. I

record my observations on the appearance of the tumour before and after passage through mice and I describe the interactions that took place between the host and the tumour and that were manifest by the populations of murine cells that were attracted into the tumour or by the presence of collagen or vascular tissue of host origin.

In Chapter 2 I describe the results of experiments that were designed to influence the rate of tumour growth in the nude mouse; i.e the size of the inoculum, the presence of co-inoculated normal or malignant cells.

In Chapter 3 I present the results of experiments done to study the effect of hormones on the growth of human melanomas in nude mouse.

In Chapter 4 I discuss the experiments that I have done to investigate the phenomenon of metastasis in the nude mouse and to define the experimental conditions that influence the development of this complication.

In Chapter 5 I report a series of experiments performed with one particularly interesting cell line that elicited an intense desmoplastic response from the host and subsequently regressed, only to recur in the same animal, at a much later stage and with a greatly accelerated growth rate. On a number of occasions repeated passage of this tumour in nude mice elicited mixed murine/human tumours and, in some instances, pure murine tumours.

At the end of the thesis I have added an appendix in which I describe the cell lines that I have used and the technical procedures and protocols that we used in the prosecution of the work recorded in this thesis.

CHAPTER 1

CHAPTER 1

GROWTH OF MELANOMAS IN THE ATHYMIC NUDE MOUSE

In 1969 Rygaard and Povlsen published the first report of the successful growth of a human tumour as a xenograft in an athymic nude mouse. Since that time an extensive literature has accumulated to testify to the interest that the nude mouse-xenograft model has generated amongst clinical oncologists and tumour biologists. There is no doubt that the use of these mutants offers exciting opportunities for achieving a deeper understanding of the neoplastic process and of the way in which the in vivo phenotype of human malignant cells is expressed or can be modulated.

A number of comprehensive reviews (Rygaard,1973; Stuttman,1977; Kindred, 1978), monographs (Rygaard,1969, 1974, 1981; Panteloris,1971; Giovanella and Stehlin, 1973; Wortis,1974; Lozzio et al.,1976b; Gullinno et al., 1976; Rygaard and Povlsen,1974, 1976; Povlsen,1977; Ward et al.,1977; Brooks et al., 1979; Fogh, and Giovanella,1978; Azar et al.,1980; Detre S.I.,1980; Sharkey and Fogh,1984) and published proceedings of symposia (Rygaard and Povlsen, 1974; Nomura et al.,1977; Houchens and Ovejera, 1978; Sparrow,1980; Bastert et al.,1981; Sordat,1982) have recently appeared to provide useful accounts of the immunology of nude mice and rats and of the experimental approaches that have been made possible by the availability of these animals. It would thus serve little purpose if I were to present an extensive documentary review of the literature myself. I shall, therefore, restrict my comments to those of a general nature regarding the sort of work that has been done using the nude mouse model and the way in which my own studies complement the results that are already available. In the first place, it is apparent that many reports in the literature have sought to do no more than record the remarkable fact that human tumours will grow in an animal that is phylogeneti-

cally far removed from man and, moreover, will express many of the attributes that they demonstrated in the original human host. In the introduction to many papers in this category, one frequently finds the justifying hope that the work to be reported will lead to the development of assay systems for measuring sensitivity to radiotherapy, cytotoxic agents or hormonal therapy, thus providing the oncologist with a sound basis for the treatment of the malignant disease that affects an individual patient. The approach is similar to that in which antibiotic sensitivity data assist in the management of infectious disorders. I think it is fair to say that, while such reports frequently provide descriptive information that is interesting they have contributed little of fundamental value, and the hopes that nude mice would provide "culture media" for sensitivity assays have not been realized.

In the second place, and more recently, the results of somewhat more interesting studies have appeared in the literature. As an example of these experiments one may cite studies in which nude mice have been used to assay tumourigenicity. It has now been abundantly documented that normal cells, when inoculated subcutaneously as a single cell suspension, will not form a progressively growing tumour whereas malignant cells frequently will do so. Anchorage-independent growth in vitro and tumorigenicity in the nude mouse have now become standard experimental criteria for the definition of the malignant state. It has thus been possible to identify, at a useful molecular level, genetic factors or other circumstances that influence or determine the expression of the neoplastic phenotype. The experiments of such persons as Thorgeirsson et al.,(1985); Graham and van der Eb,(1973); Barlogie,(1980); Stiles and Kawahara,(1978); Freedman and Shin,(1978); Ossowski et al., (1973); Geder et al.,(1976) and Freedman et al.,(1976) demonstrate the elegant way in which these approaches can be used.

Finally, recent major contributions to the understanding of the biochemistry and genetics of the malignant cellular phenotype have come from the

laboratories of cellular biologists and experimental pathologists. These advances in knowledge have stimulated a large number of systematic and quantitative studies of xenografted human tumours in the likely hope that ideas born of basic *in vitro* research will be testable in the intuitively more authentic, *in vivo* nude mouse model. Invasion and metastasis, angiogenesis, para-neoplastic syndromes and the complex interactions that take place between tumour and host are some of the many aspects of oncology that can only really be studied *in vivo*. What better model for studying them than the immunodeficient animal? Here we have a system that provides the experimental convenience of tumour bearing hosts whose characteristics are well defined and that are available in sufficient numbers to provide adequate statistics and controls. Although, for the most part, reports that fall into this group are still largely descriptive, many of them do provide the sort of consistent, quantitative data that are required to establish a sound basis for further more innovative research.

It is to this latter category that I hope the work I describe in this chapter belongs for in it I describe the *in vivo* growth characteristics of seven human melanoma lines when these were injected into nude mice. I have attempted to document the characteristics of these cells and the *in vivo* expression of their neoplastic potential in definitive, quantitative and qualitative terms. Where I have been successful I have made observations that have served as a point of departure for some of the other work that I report in this thesis. I refer, in particular, to experiments that have shown that it is technically feasible to obtain individually consistent and reliable quantitative data on the kinetics of melanoma cell growth *in vivo*; that tumorigenicity and growth rate are dependent upon the size of the tumour inoculum and, in certain instances, on the sex of the recipient animal; that certain human melanomas metastasize widely in the nude mouse whereas others do

not; certain tumours elicit an intense host response whereas in others this response is barely detectable; and, finally, that human melanoma cells growing in vivo show diverse behaviour patterns and this heterogeneity is, in itself, an interesting subject for further study.

In this chapter I describe the in vivo growth characteristics of the tumours that resulted when cells from these seven human melanoma cell lines were injected into athymic nude mice.

The work that comprises this thesis has been done with seven human melanoma cell lines that had already been developed in our laboratory. My choice of melanomas for this study was dictated by several factors. Aside from the fact that they were already available, these tumours are well known for the ease with which they can be cultured in vitro (Romsdahl and Hus, 1972; Liao et al., 1975; Gerner et al., 1975; Giovanella et al., 1976; Fidler and Nicolson, 1976; Hoal et al., 1982; McCormick et al., 1983), and they can usually be successfully xenotransplanted to athymic mice. The many melanoma cell lines that have been developed and studied by other researchers, testify to the convenience of these cells, and the many published reports provided a sound basis, in the literature, from which my own work could proceed. Malignant melanomas are also known for their ability to invade normal tissues and to spread widely. These cells thus constitute a useful model for research into the biology of metastasis.

Cell lines

The melanoma cell lines were derived from biopsies taken from metastatic deposits in patients operated upon in Groote Schuur Hospital. The characteristics of the individual cell lines, the techniques used to derive them and the manner in which they were maintained in culture and in storage have been described (Wilson and Dowdle, 1978; Hoal et al., 1982) (Appendix A1-A6).

Briefly, the fragments of tissue were placed in vials containing sterile transport medium (RPMI 1640) and transferred to the laboratory. On arrival they were disrupted by gentle teasing, by treatment with trypsin and collagenase. The resulting suspensions of single cells, small clumps and debris were plated in plastic tissue culture petri dishes in RPMI 1640 medium supplemented with 10% heat inactivated foetal bovine serum and antibiotics (RPMI-10). All cultures were incubated at 37 ^{o}C in a humid atmosphere of air and 5% carbon dioxide.

In the early stages, the cultures were regularly examined by phase contrast microscopy for the growth of melanoma cells and various strategies were used to select against overgrowth by fibroblasts. These usually exploited the fact that melanoma cells adhered less tenaciously to the plastic substrate than did fibroblasts and could thus be recovered, in enriched proportions, by selective trypsinization or by differential adherence.

Once established, the cultures were maintained in RPMI-10 and passaged at intervals dictated by confluence and by the condition of the medium. Repeated passage may give selective advantage to cellular variants that arise in vitro. To avoid the effect of this genetic drift, I used, for most of my experiments, cells derived from stocks that had been frozen down from a single passage or from closely consequential passages. Cells were frozen in RPMI-1640 medium containing 10% foetal calf serum and 10% dimethyl sulfoxide (Farrant et al.,1974).

All of the cells grew as adherent monolayers with the characteristics

summarized in Table 1.1 and Table A.7 in the Appendix.

Mice

Athymic nude mice were bred in the U.C.T. Animal Unit from breeding stocks imported in June 1980 and provided by Dr. B.C. Giovanella of the Cancer Research Laboratory, St. Josephs Hospital, Houston, Texas, USA. The strain was developed by Hansen (1977) by introducing the "nude" gene (nu) and the x-linked immune deficiency gene (xid) into N-NIH(S) outbred stock. This strain thus has combined immunodeficiency (Azar et al., 1980) and has proved extremely susceptible to grafts of human malignant tumours.

The animals were housed in sterile plastic cages covered with non-woven spun polyester filter hoods (Laboratory Products Inc., Mainwood, New York) and sealed with adhesive tape. Clean cages were provided once weekly. Access to the colony was restricted to essential personnel and all manipulations were conducted in a horizontal laminar flow hood under strictly aseptic conditions. All supplies entering the housing area were sterilized either by steam or ethylene oxide.

The mice were fed a commercially prepared rodent food (mouse pellets, Epol (Pty) Ltd., Cape Town). The diet of lactating females and weanlings was supplemented with an autoclaved 3:1 (w/w) mixture of whole wheat bread and milk powder (Lactogen Baby Formula, Food and Nutritional Products (Pty) Ltd., Randburg). To compensate for the possible loss of vitamins during autoclaving, I added a multivitamin supplement to the drinking water (0.5ml/litre of Pancebrin; Lilly Pharmaceuticals). Cefamandole nafate (27 mg/litre, Lilly Pharmaceuticals) was added to the drinking water as prophylaxis against infection. To avoid any possible effects of antibiotics on tumour growth I discontinued the Cefamandole seven days before the start of any experiments.

The nu gene was maintained by the mating of homozygous nu/nu male mice with heterozygous nu/+ females. Half the progeny were thus homozygous nu/nu;

Table 1.1

FEATURES OF MELANOMA CELL LINES

Cell Line	Doubling time (hrs)	Modal Chromosome No.	Pigment in vitro
UCT-Mel 1	41	74	+
UCT-Mel 2	50	61	+
UCT-Mel 3	58	72	-
UCT-Mel 4	52	100	-
UCT-Mel 5	58	72	-
UCT-Mel 6	33	81	-
UCT-Mel 7	52	65	-

their growth rate was improved by removing nu/+ litter mates one week before weaning. Mating between homozygous pairs was not practical since the homozygous females nursed poorly. As far as was possible with our small isolated colony, I minimised inbreeding in an attempt to preserve vigour and to prevent decline in litter size. Young were weaned at 21 days of age.

The breeding data for the colony for the years 1981 to 1985 are summarized in Table 1.2. The average litter size was approximately 7. Virtually all of the nudes were weaned, with a very low pre-weaning mortality. The productivity was 1.63 young/female/week with a generation gap of 3 months and each female produced, on average, 2.5 - 3.9 litters every six months.

I attribute much of the success that I have had with the colony to the strict control that I exercised over the physical environment of the animals. Under germ-free conditions in a protected milieu, the lifespan of the nu/nu mice was the same as that of normal laboratory mice.

Periodic monitoring of the animals for disease was done according to the recommendations of in the British Medical Research Council. Occasional instances of bacterial contamination by organisms such as Staphylococcus epidermitis, Bacillus spp and Lactobacillus, were recorded. In these cases infected animals and "contact" animals were immediately transferred out of the colony.

Growth curves of the mice from the time of weaning until they attained adulthood at the age of 8-10 weeks are shown in Figure 1.1.

Histological sections of lymph nodes (Fig 1.2) showed the typical paracortical depletion of thymic deficiency.

Table 1.2

STRAIN NAME	YEAR	PWM %	MEAN LITTER SIZE	PRODUCTIVITY (YOUNG/♀/WEEK)	NO. OF LITTERS 4-6 MONTHS
NIH II (nu/nu) (outbred)	1981	3.2	7.0	1.5	2.8(F1)
	1982	1.3	6.2	1.9	2.5(F2-F4)
	1983	0.7	6.9	1.2	3.9(F5-F8)
	1984	3.5	7.7	1.5	3.8(F9-F12)
	1985	3.2	8.5	2.3	3.7(F13-F15)

PWM = Pre-weaning mortality.

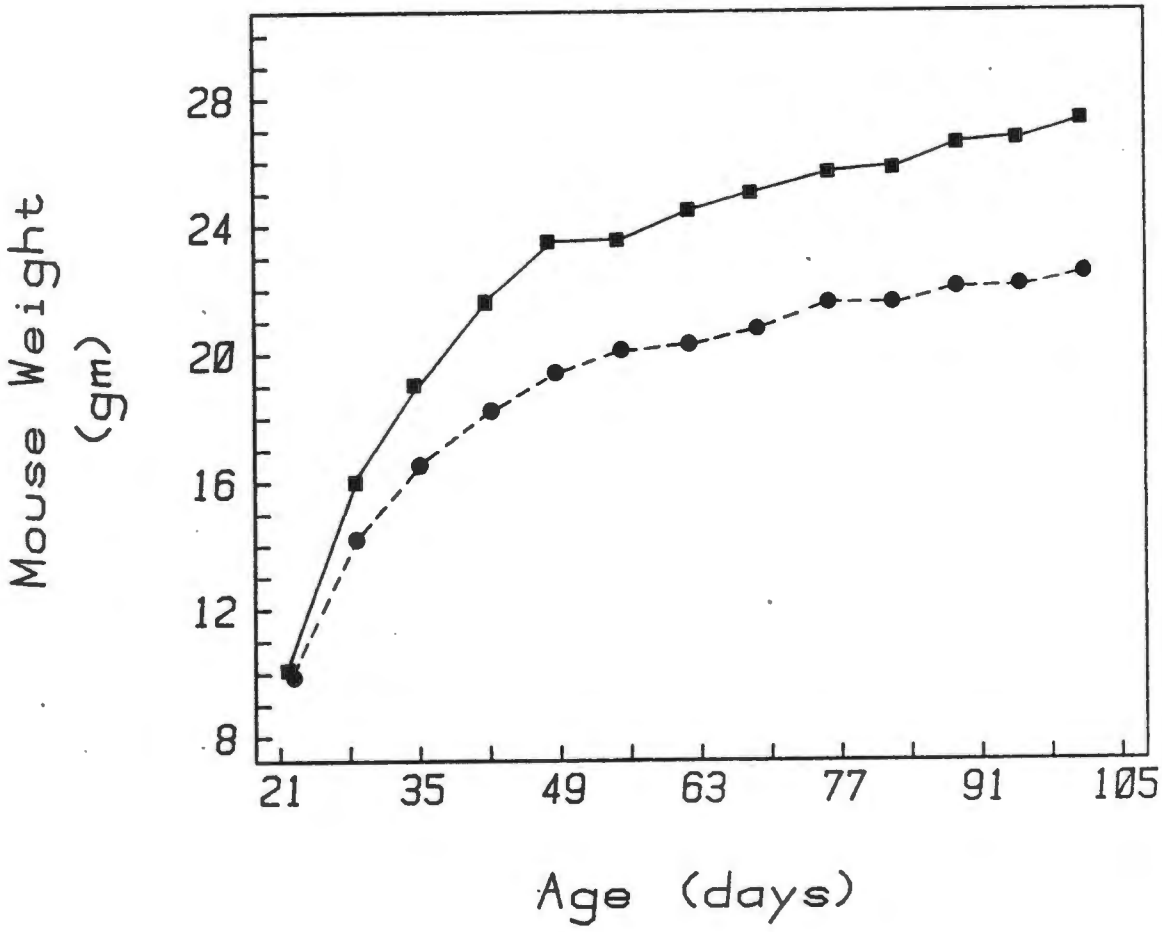


Figure 1.1

Weight of nude mice as a function of age

Mice were weaned at 21 days of age and weight measured once a week. The points on the graph were obtained by plotting the average body weight of 5 mice. The mice illustrated are as follows: males (■——■); females (●----●).

FIGURE 1.2

Figure 1.2

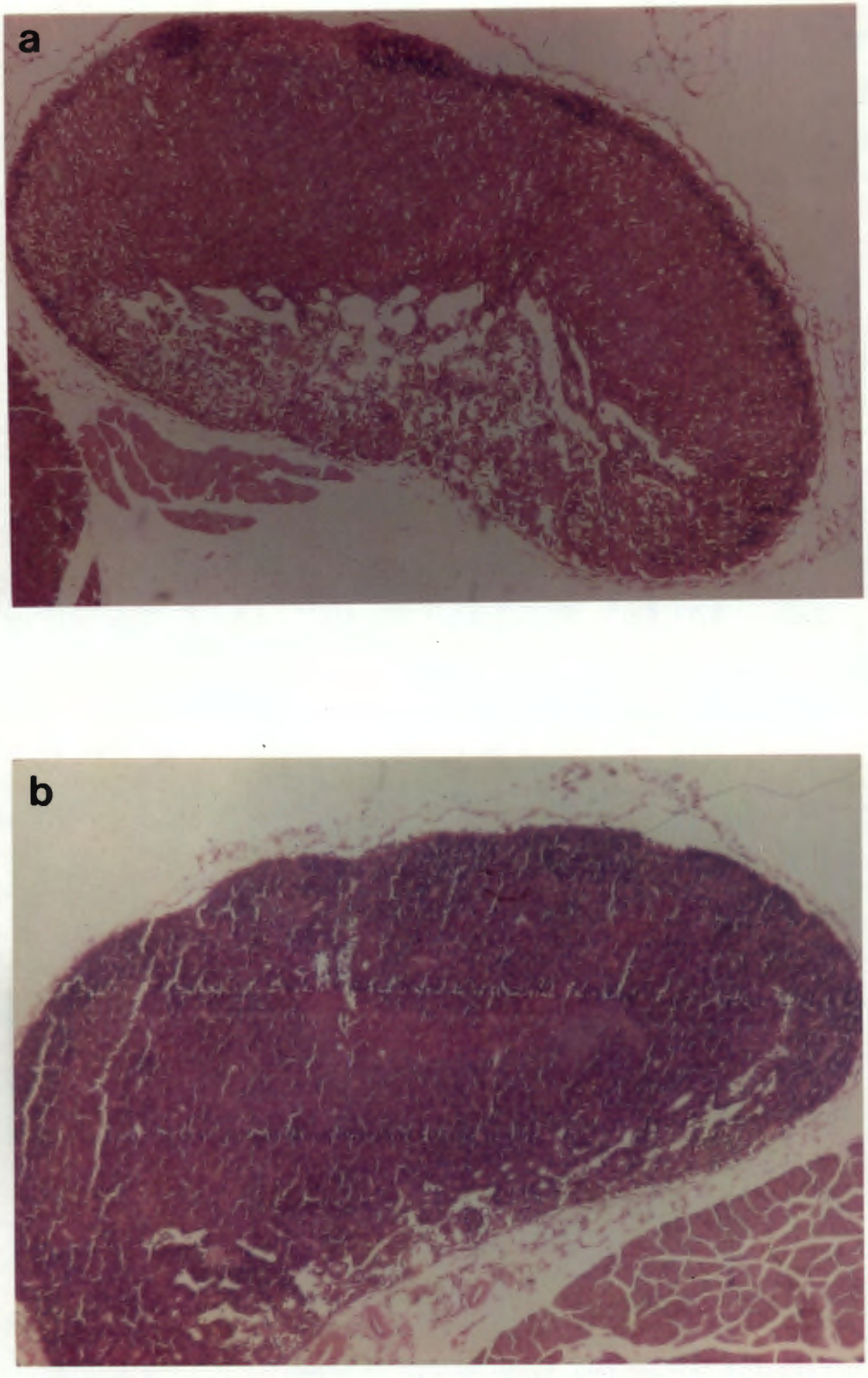
Comparative histological cross-sectional areas of lymph nodes

Histological sections of representative lymph nodes were stained according to conventional histological techniques (H & E). The lymph nodes are as follows:

a) Axillary lymph node of N:NIH(S) - nu/nu mouse shows a paracortical lymphocyte depletion.

b) Axillary lymph node of an immunocompetent Balb/c mouse shows a normal cortical and paracortical lymphocytic distribution.

Figure 1.2



EXPERIMENTAL PROTOCOL

Cells for inoculation into the mice were harvested from the tissue culture dishes by washing with pre-warmed (37 °C) serum-free medium and then incubating under warmed TBS containing 0.06% trypsin and 0.02% EDTA. Intermittent inspection by phase-contrast microscopy and gentle tapping ensured that the monolayers were not exposed to the trypsin-EDTA solution for any longer than was necessary to achieve adequate release of viable cells. When this was complete (usually within 2-5 min) the trypsin was neutralized by the addition of foetal calf serum to a final concentration of 7.5%. The cells were then pelleted and washed once with serum-free medium. They were then resuspended in serum-free RPMI medium; checked by trypan-blue exclusion for viability, and then diluted to give the required number of viable cells in an inoculum of 0.1ml.

Working under sterile conditions in a laminar flow hood, I inoculated cells subcutaneously into the inter-scapular region of 6-8 week-old animals. This was best achieved by having an assistant hold the mouse steady by grasping a skin fold at the back of the neck with the left hand and the haunches with the right hand. The mouse, extended between the two hands of the assistant, was then held firmly in the prone position on the working surface. I then lifted, between finger and thumb of my left hand, a small fold of skin in the middle of the dorsum of the back and through this I introduced a 38mm x 26 gauge hypodermic needle attached to the syringe containing the cell suspension. The needle was advanced cephalad, taking care to remain in the plane of the subcutaneous tissues. The entire inoculum of 0.1ml was delivered slowly at the tip of the needle and at the end of the needle track. Using this technique I could inject reproducible numbers of cells into a confined region without depositing any along the needle track or having them regurgitate through the inoculation site.

The animals were then observed for up to approximately 10 months for the

appearance of tumours. The mice were weighed once a week and any tumours that developed were measured. The tumour volume was calculated as the product of three major diameters. Generally speaking the tumours assumed a spherical or elliptical shape and were confined to a single subcutaneous site. Measurement was thus straightforward.

In most experiments - and particularly those in which we were studying metastatic spread of the primary tumour - tumours were excised when a certain desired mass had been achieved. This was done under ether anaesthesia. After brief swabbing of the skin with 70% alcohol an elliptical incision was made and the tumour was removed with the enclosed ellipse of skin. In most cases the tumour was well defined and very easily removed from the local site of primary growth with little, if any, evidence of invasion. The skin wound was closed with interrupted stitches using 00 Dermalon and an atraumatic needle.

In approximately 10% of cases where tumours were removed from mice that had been inoculated with UCT-MEL 3 cells, local recurrence of tumour growth was observed. This was rarely seen with tumours derived from other cell lines. It is of interest to note that, despite the immunodeficient status of the mice, wound infection did not occur and the wounds all healed remarkably well.

Representative portions of each tumour were placed into buffered formal saline for histology. Regions of the tumour that appeared viable were put into vials containing sterile tissue culture medium and re-inoculated into tissue culture dishes or re-implantated as fragments, into mice. Small pieces of the tumour were also snap-frozen in liquid nitrogen for enzymatic analysis or cryopreserved by gradual freezing in RPMI-1640 medium containing 10% foetal calf serum and 10% dimethyl sulfoxide (Farrant et al., 1974).

Analysis of tumour tissues

An estimate of the relative contribution of human and murine cells to the

tumour mass was obtained by agarose gel electrophoresis of an homogenate of the tumour tissue followed by zymographic localization of the bands of lactate dehydrogenase (LDH) activity. This was done using standard procedures (Nichols and Ruddle, 1973; Detter et al., 1968; Krog, 1976). Human and murine LDH were readily distinguishable by differences in electrophoretic mobility (Fig. 1.3)

Formalin-fixed samples were embedded in paraffin, sectioned and stained according to conventional histological techniques (Mayer's haematoxylin and eosin).

Mathematical analysis

Tumour growth was analysed by fitting the Gompertzian function:-

$$V(t) = Vmax.\exp\left[-\frac{\alpha}{\beta}\cdot e^{-\beta t}\right] \quad \text{.....eq. 1}$$

to the tumour volume, V(t) plotted as a function of time, t.

The parameters Vmax, alpha and beta were derived as recommended by Rofstad, Fodstad and Lindmo (1982) by applying successive linear regression analysis to the three following linear forms of equation 1.

$$\text{(i)} \quad \frac{1}{V(t)} \cdot \frac{\Delta V(t)}{\Delta t} = -\beta \ \ell n\ V(t) + \beta \ \ell n\ Vmax$$

$$\text{(ii)} \quad \ell n \left[\ell n \frac{Vmax}{V(t)} \right] = -\beta t + \ell n \left(\frac{\alpha}{\beta} \right)$$

$$\text{(iii)} \quad \ell n\ V(t) = -\frac{\alpha}{\beta}\cdot e^{-\beta t} + \ell n\ Vmax$$

FIGURE 1.3

Figure 1.3

Lactate dehydrogenase analysis on human tumours removed from nude mice

Mice were inoculated subcutaneously with either 10^6 or $5x10^6$ UCT-Mel 7 cells (*in vitro* passage numbers 26', 29', 30'). These tumours grew exponentially for a period of approximately 80 days after which growth ceased and tumours regressed. Four to 7 months after initial inoculation of these tumour cells tumours started growing once more. They now grew vigorously showing exponential growth kinetics. At this stage tumours were removed and small explants were implanted subcutaneously into new animals. The tumours were homogenized and LDH activities determined.

Samples of tumour homogenates were as follows:-

Channel 1, 2 and 3 - UCT-Mel 7 tumour tissue homogenate from passage 6, 7and 7 respectively, showing LDH enzyme bands of both human and mouse origin.

Channel 4 - mouse liver homogenate, showing LDH enzyme band of mouse origin.

Channel 5 - human breast homogenate, showing LDH enzyme band of human origin.

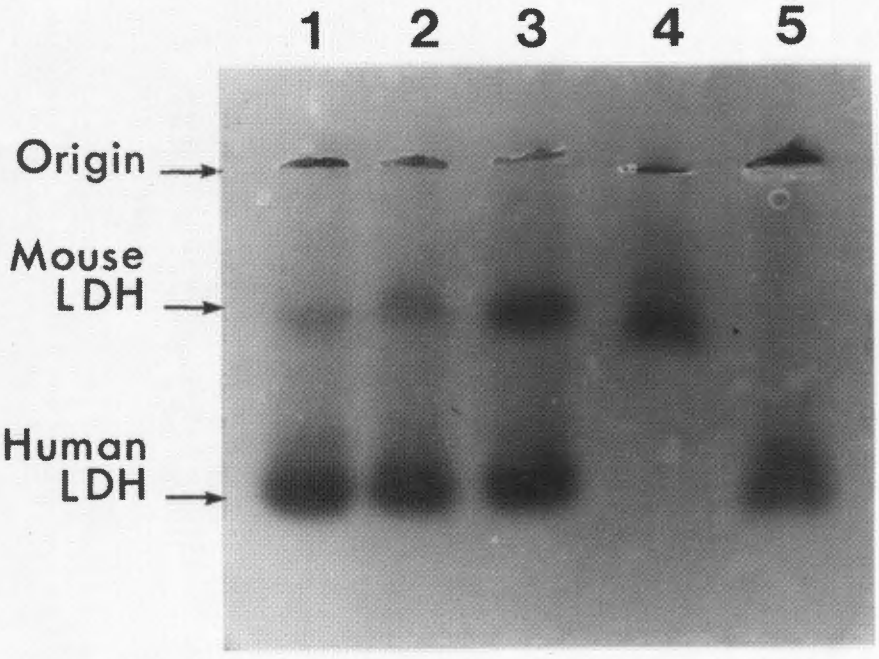


Figure 1.3

In these equations, alpha represents the first-order rate constant that describes the increase in growth rate with increasing tumour size; beta represents the superimposed negative rate constant that describes the decrease in growth rate with increasing size; and Vmax represents the theoretical maximum tumour volume.

Since the Gompertzian function gives a curvilinear relationship between lnV(t) and t, one cannot define a global doubling time, (Td)^ for the tumour. I have calculated, instead, the instantaneous doubling time at a particular volume, V(t). Since measurements of V(t) in the vicinity of $200mm^3$ were usually reliable, I give for each tumour, the Td calculated for this volume as follows:

$$Td(200) = -\frac{1}{\beta} \ell n \left[1 - \frac{\ell n\, 2}{\ell n\ (Vmas/200)} \right]$$

Since I only examined the mice once weekly, I was unable to determine the delay time between inoculation of the cells and appearance of the tumour, with a precision of more than approximately 7 days. In any event, the first appearance of a minute tumour would have been rather a difficult event to time accurately.

The "delay time", (Tdel) for each tumour was therefore derived by calculating the length of time taken, after inoculation of the tumour cells, to reach a volume of $100mm^3$ on the Gompertzian curve. This was calculated by substituting 100 for V(t) in equation 1 and solving for t. Thus

$$Tdel = \frac{-1}{\beta} \cdot \ell n \left[-\frac{\beta}{\alpha} \cdot \ell n\ (100/Vmax) \right]$$

RESULTS

Kinetics of tumour growth

Of the six melanoma cell lines that I inoculated, all were tumorigenic with the exception of UCT-Mel 6. Cells from this line failed to form tumours despite several attempts to induce growth with larger inocula and co-inoculation of other cells.

When tumours did develop, their growth was mathematically well described by considering the volume of the tumour as a Gompertzian function of time (Figs. 1.4 - 1.11).

Thus $V(t) = Vmax.\exp(-\alpha/\beta.\exp(-\beta t))$.

Here $V(t)$ is the volume of the tumour in mm^3 at time t; $Vmax = \lim V(t)$ (the theoretical maximum tumour volume) and α and β are constants. The Gompertz parameters that I calculated as described in the Methods section are presented in Table 1.3.

The time that elapsed between inoculation and the interpolated theoretical volume of $100mm^3$ (i.e. the delay time to $100mm^3$) varied from 12 to 22.2 days for an inoculum size of $5x10^6$ cells and from 22.8 to 33.7 days where $1x10^6$ cells were injected. When, in a different series of experiments, 10^7 and $5x10^7$ cells for UCT-Mel 1 and UCT-Mel 2 were injected, a similar shortening of the delay time was seen with the larger inocula. It is noteworthy that the delay times in these experiments were similar (or in some cases greater) than those seen when ten-fold less cells were given in the first set of experiments. When 10^7 UCT-Mel 2 cells were inoculated, for example, a mean delay time of 29.7 days was recorded as opposed to 24.8 days for an inoculum of 10^6 cells.

Theoretical instantaneous doubling times at a volume of $200mm^3$ were calculated for 5 of the 6 tumours that grew. The 6th line (UCT-Mel 7) reached a plateau at approximately $200mm^3$. Values for this parameter varied from 4.9 to 10.7 days. In general the inoculum size had no consistent effect upon the

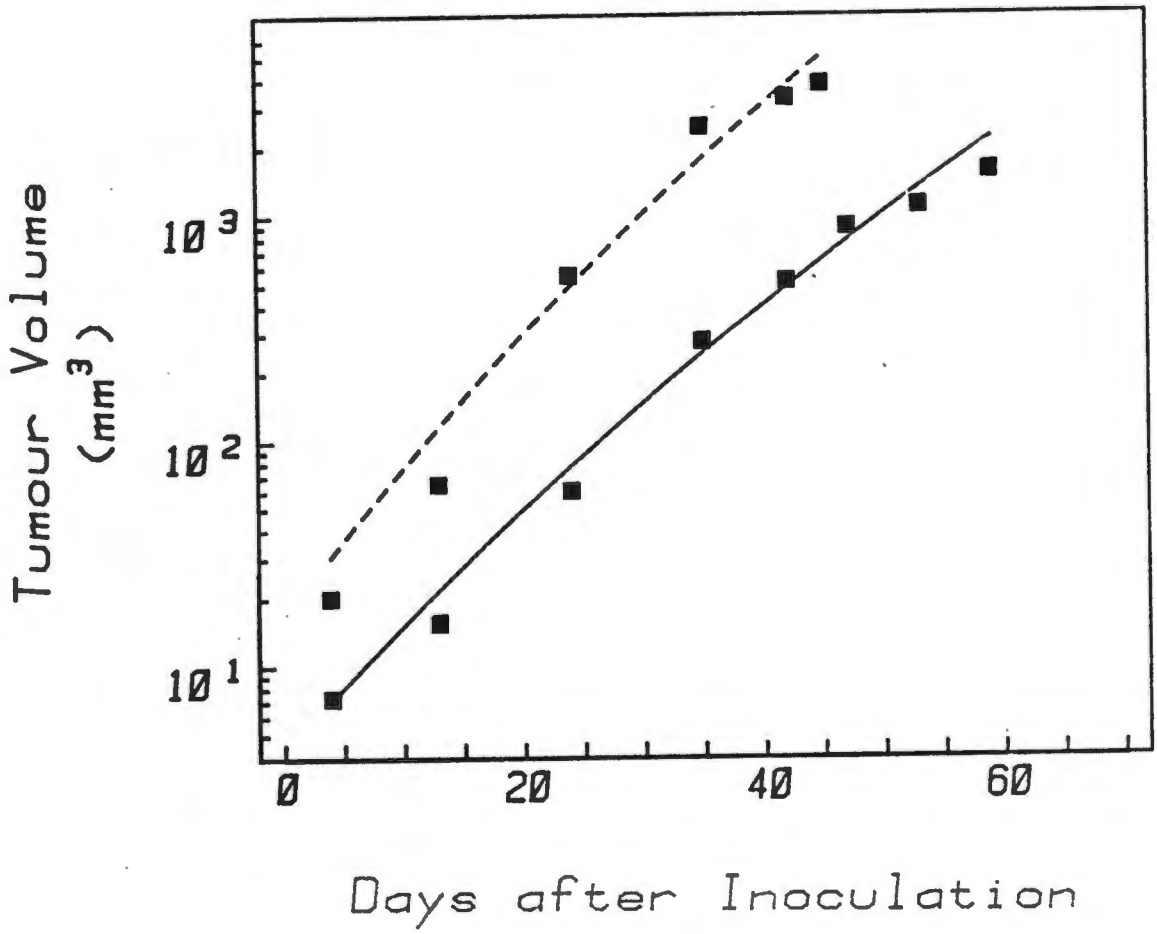


Figure 1.4

Growth of human melanomas in nude mice

Mice were inoculated subcutaneously with 10^6 (■——■) or $5x10^6$ (■----■) UCT-Mel 1 cells (passage number 65') on day 0 and the tumour volume determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values of 6 mice in the case of 10^6 cells and 5 mice in the case of $5x10^6$ cells.

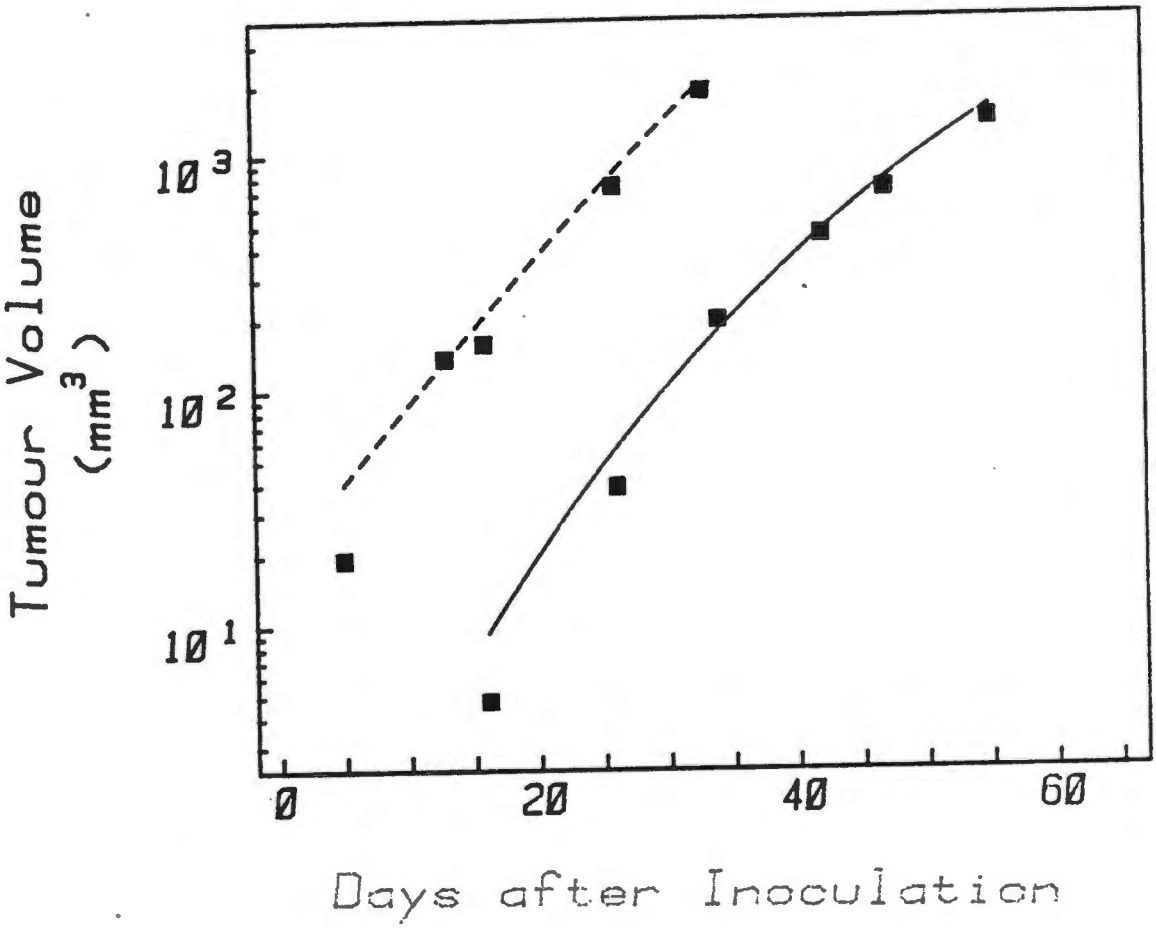


Figure 1.5

Growth of human melanomas in nude mice

Mice were inoculated subcutaneously with 10^7 (■——■) or 5×10^7 (■----■) UCT-Mel 1 cells passage number 65' on day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice.

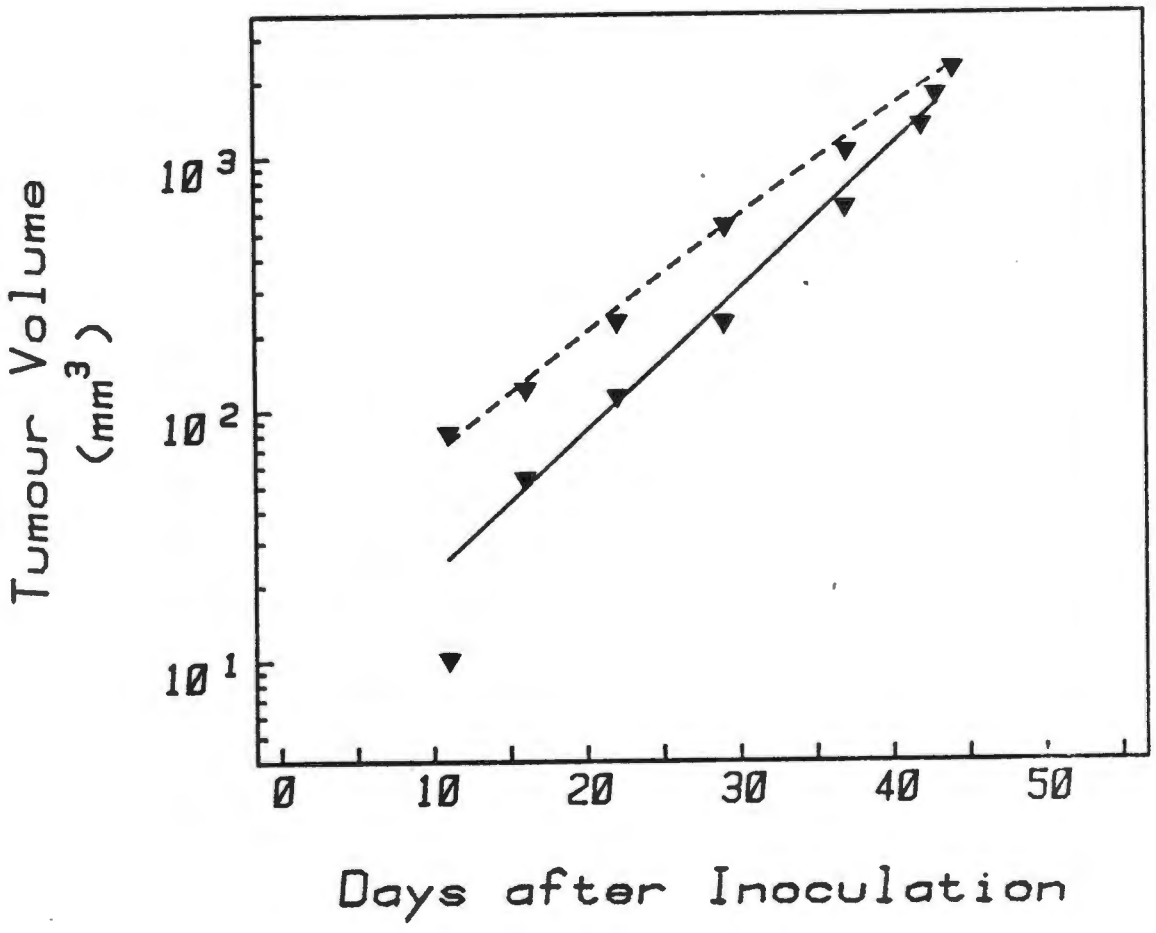


Figure 1.6

Growth of human melanomas in nuce mice

Mice were inoculated subcutaneously with 10^6 (▼——▼) or 5×10^6 (▼----▼) UCT-Mel 2 cells (passage number 127') on day 0 and the tumour volume determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice.

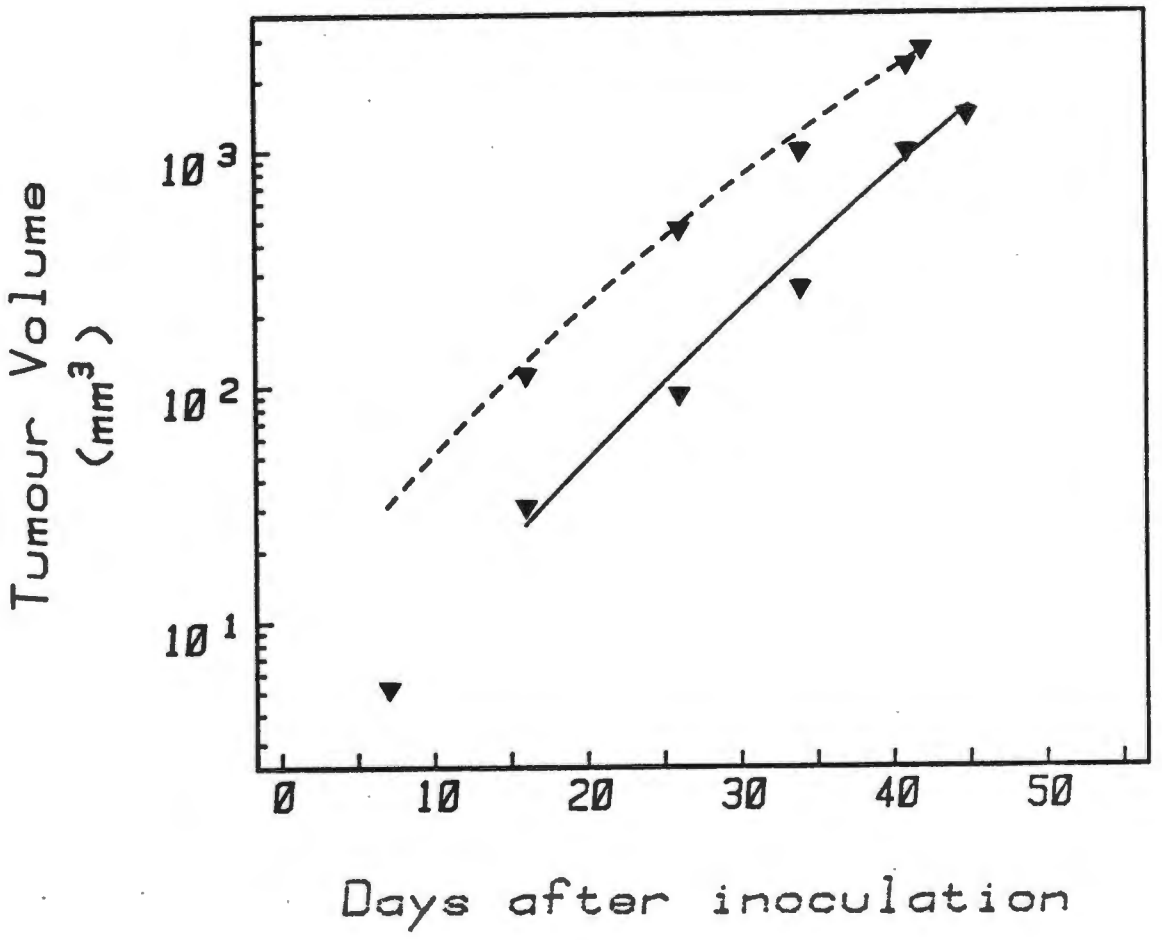


Figure 1.7

Growth of human melanomas in nude mice

Mice were inoculated subcutaneously with 10^7 (▼——▼) or 5×10^7 (▼----▼) UCT-Mel 2 cells (passage number 127') on day 0 and the tumour volume determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice.

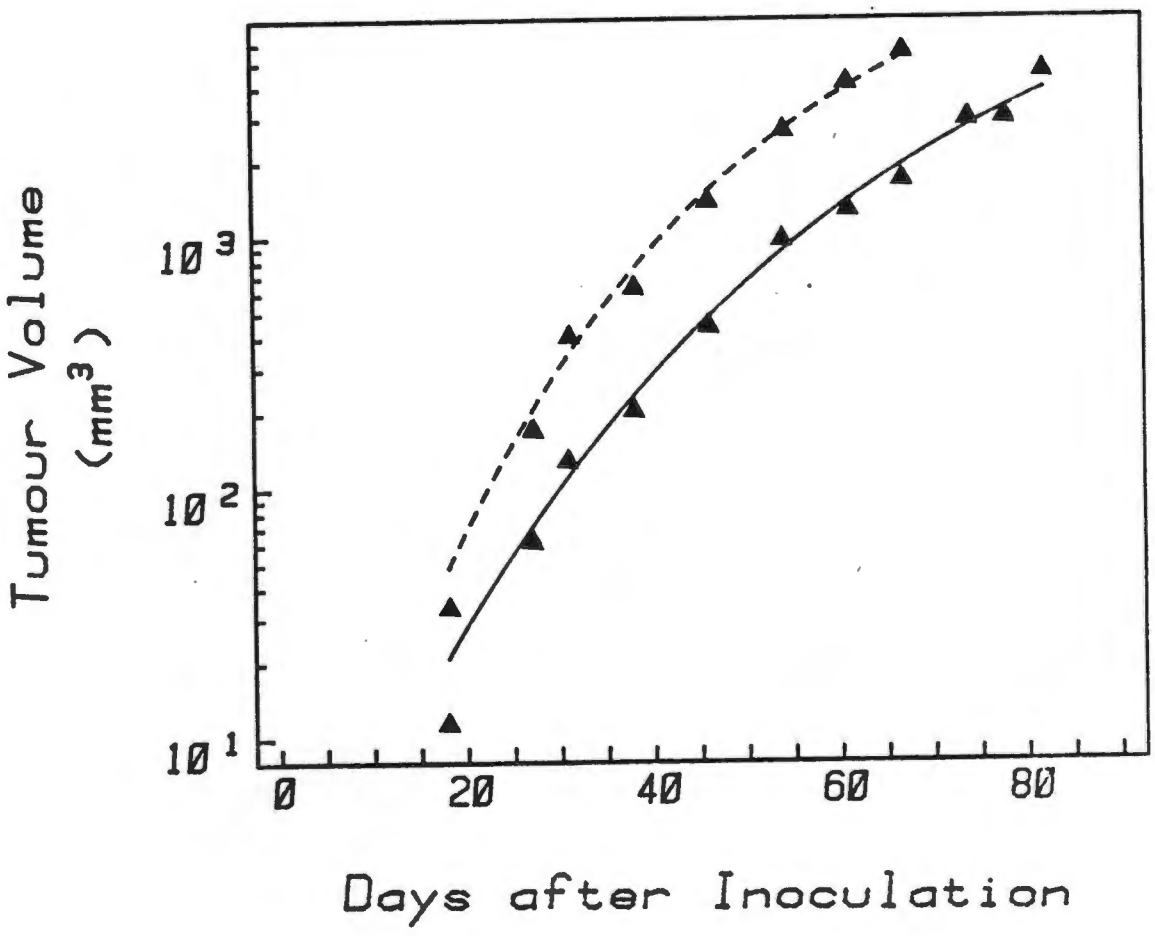


Figure 1.8

Growth of human melanomas in nude mice

Mice were inoculated subcutaneously with 10^6 (▲——▲) or 5×10^6 (▲----▲) UCT-Mel 3 cells (passage number 69') on day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice in the case of 10^6 cells or 3 mice in the case of 5×10^6 cells.

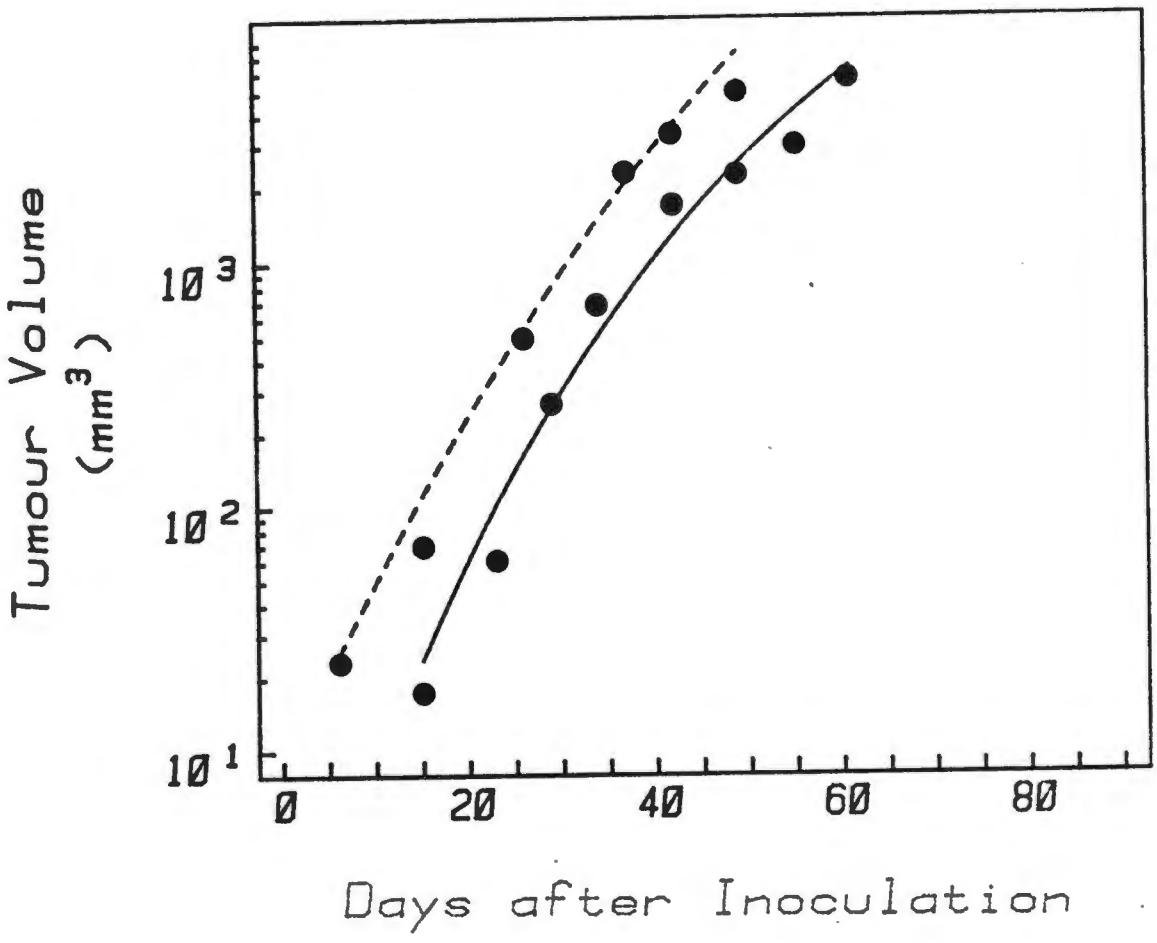


Figure 1.9

Growth of human melanomas in nude mice

Mice were inoculated subcutaneously with 10^6 (●——●) or 5×10^6 (●-----●) UCT-Mel 4 cells (passage number 20') on day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice.

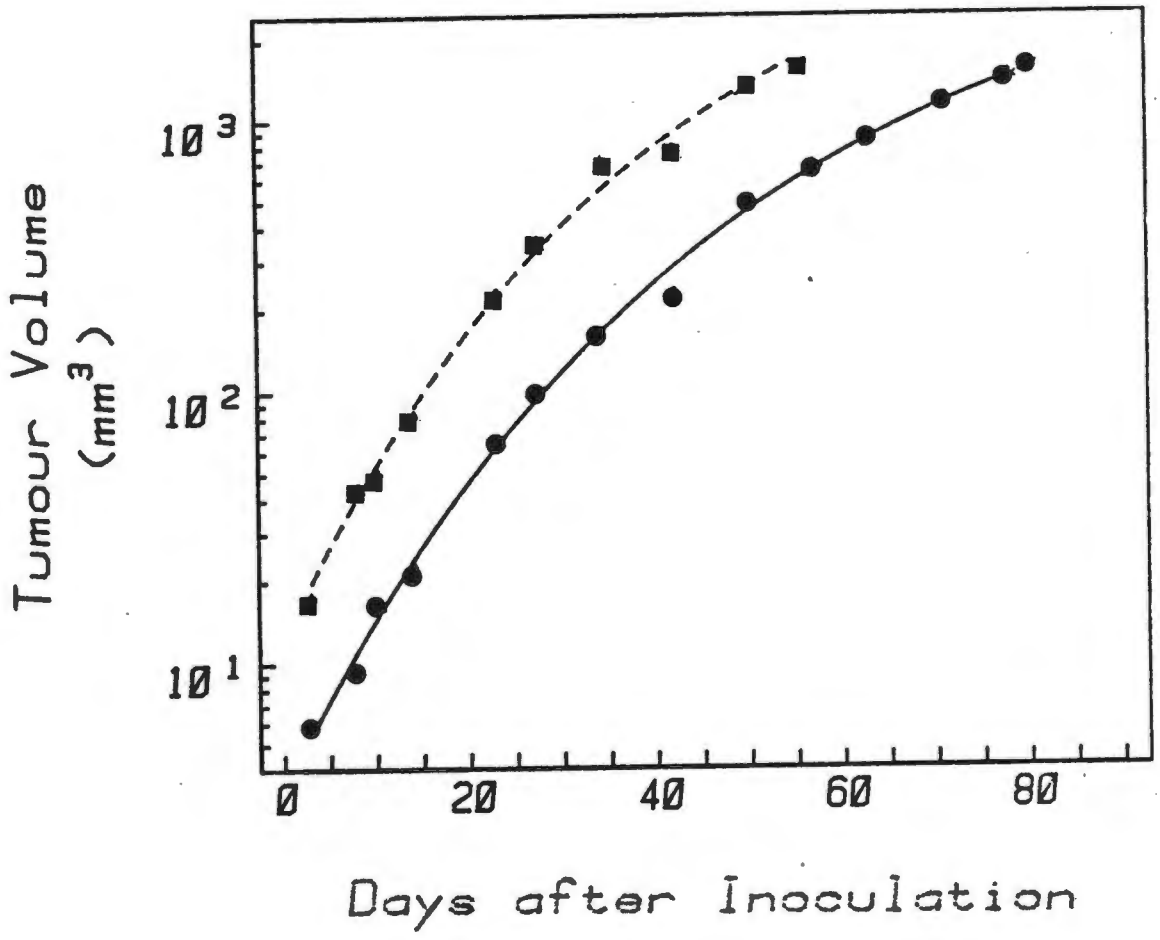


Figure 1.10

Growth of human melanomas in nude mice

Mice were inoculated subcutaneously with 10^6 (●——●) or $5x10^6$ (■- - - -■) UCT-Mel 5 cells (passage number 27') on day 0 and the tumour volume determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice.

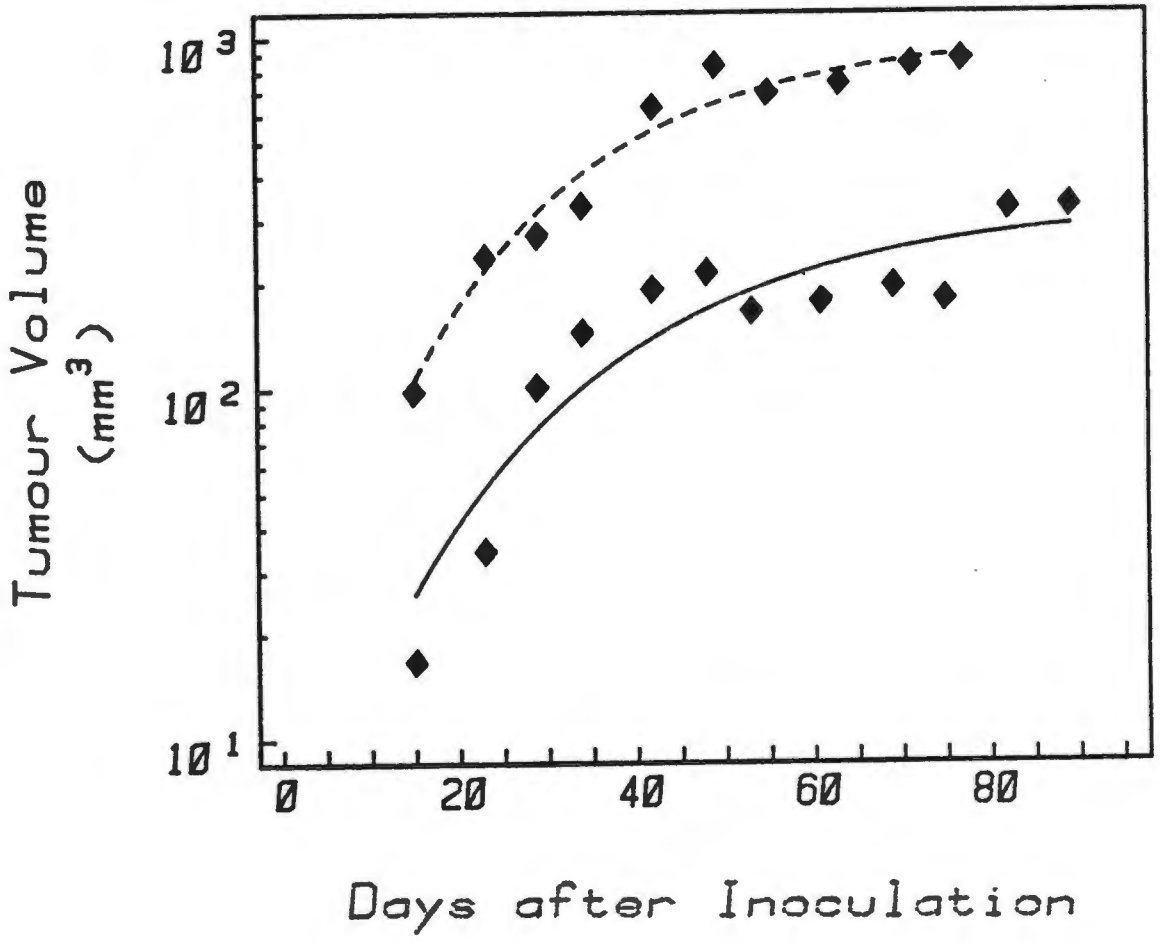


Figure 1.11

Growth of human melanomas in nude mice

Mice were inoculated subcutaneously with 10^6 (◆——◆) or 5×10^6 (◆- - -◆) UCT-Mel 7 cells (passage number 29') on day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice.

TABLE 1.3

CELL LINE	NO. OF CELLS	DELAY TIME $100mm^3$ (days)	α (day-1)	β (day-1)	Vmax (mm^3)	Td(days) ($V=50mm^3$)	Td(days) ($v=200mm^3$)	Td(days) ($v=1000mm^3$)
UCT-Mel 1	1×10^6	26.4	0,1245	8.18^{-3}	$5.2x10^7$	6.3	7.0	8.1
	5×10^6	12.0	0.1469	1.08^{-2}	$4.5x10^7$	4.8	5.3	6.2
	1×10^7	24.8	0.1795	$2,41^{-2}$	$4.2x10^4$	4.5	5.8	8.5
	5×10^7	14.4	0.1512	7.35^{-3}	$6.3x10^{10}$	4.6	4.9	5.4
UCT-Mel 2	1×10^6	24.8	0.1299	4.34^{-4}	-	5.3	5.4	5.4
	5×10^6	14.4	0.1232	6.91^{-3}	$4.3x10^9$	5.6	6.1	6.7
	1×10^7	29.7	0.1525	8.87^{-3}	$1.6x10^9$	4.6	5.0	5.6
	5×10^7	11.0	0.1557	1.68^{-2}	$6.7x10^5$	4.5	5.3	6.7
UCT-Mel 3	1×10^6	29.2	0.1341	2.08^{-2}	$2.5x10^4$	5.7	7.4	11.7
	5×10^6	22.2	0.1589	2.98^{-2}	$2.1x10^4$	4.1	5.4	8.6
UCT-Mel 4	1×10^6	22.8	0.1825	2.42^{-2}	$9.5x10^4$	4.0	4.9	6.8
	5×10^6	14.4	0.1659	1.43^{-2}	$5.5x10^6$	4.3	4.9	5.8
UCT-Mel 5	1×10^6	28.0	0.1418	2.16^{-2}	$5.7x10^4$	7.3	10.7	23.6
	5×10^6	15.1	0.1451	2.58^{-2}	$8.1x10^3$	5.7	8.0	15.6
UCT-Mel 7	1×10^6	33.7	0.1628	6.77^{-2}	$3.0x10^2$	-	-	-
	5×10^6	14.4	0.1067	5.97^{-2}	$8.8x10^2$	-	-	-

Td(200).

Assuming that the parameter alpha represents the fractional first order rate constant for exponential growth, i.e. $V(t) = V(o)e^{\alpha t}$, I have calculated theoretical doubling times for the inocula of 10^6 cells. In table 1.4 these are compared with the observed doubling times for the corresponding cell lines cultured in vitro. The cell generation time or doubling time in vitro was approximately 2 to 3 times less than that of the tumour doubling time in vivo. I have also included in Table 1.4, reference to observations by Spang-Thomsen et al., (1984) who derived a cell cycle time of 44 hrs for a melanoma xenograft using a technique in which radioactive thymidine was administered to the tumour-bearing nude mouse and, at the same time, measured the tumour volume doubling time from a Gompertzian analysis similar to that that I have used. Close agreement with my data is evident.

In Figure 1.12 I compare the values for Td(200), α and β that I observed with those presented by Rofstad et al., (1982). These box plots, (which provide a convenient, non-parametric means of displaying the distribution of data around the median values) show that the combination of melanoma cells and nude mice that I studied had Td(200) values that fell well within the range of those reported by Rofstad although the median values were somewhat shorter and my data showed less scatter. The data for both α and β showed overlap between the two series; once again my values were smaller, with less scatter, than those of Rofstad et al., (1982).

UCT-Mel 7 cells were unusual in that, after a period of early growth (0 - approximately 80 days) during which they followed Gompertzian kinetics, growth ceased at a plateau followed by a regression in tumour size to the point, in some cases, where only a small, barely palpable, nodule remained. Then, surprisingly, and usually 4 to 7 months after inoculation of the tumour cells, the nodule started to grow again, this time as a vigorous tumour showing simple exponential growth kinetics (Fig. 1.13).

Table 1.4

Comparison of doubling times for growth in vivo and in vitro

Cell line	Doubling time (hours)	
	In vitro (1)	In vivo (2)
UCT-Mel 1	41	134
UCT-Mel 2	50	128
UCT-Mel 3	58	124
UCT-Mel 4	52	91
UCT-Mel 5	55	117
Spang-Thomsen & Vindeløv	41.4 - 43.9 (3)	137 - 187

(1) Cells were cultured, with daily medium replacement in RPMI-1640 supplemented with 10% foetal calf serum and antibiotics. Cells in duplicate cultures were counted every second day during the period of exponential growth.

(2) Calculated from the values for the Gompertzian parameter, alpha, presented in Table 1.3 on the assumption that this represents the fractional first order rate constant for growth as a function of time.

$$\text{i.e. } V(t) = V(o).\ \exp(\alpha t)$$

(3) These results were obtained by the authors (Spang-Thomsen & Vindelov, 1984) by administering ^{3}H-thymidine to tumour bearing mice and counting labelled mitoses in biopsies taken at intervals thereafter. Their data for in vivo volume doubling times were derived, as were mine, for tumours ranging in size from 90 to larger than 255mm^{3}.

FIGURE 1.12

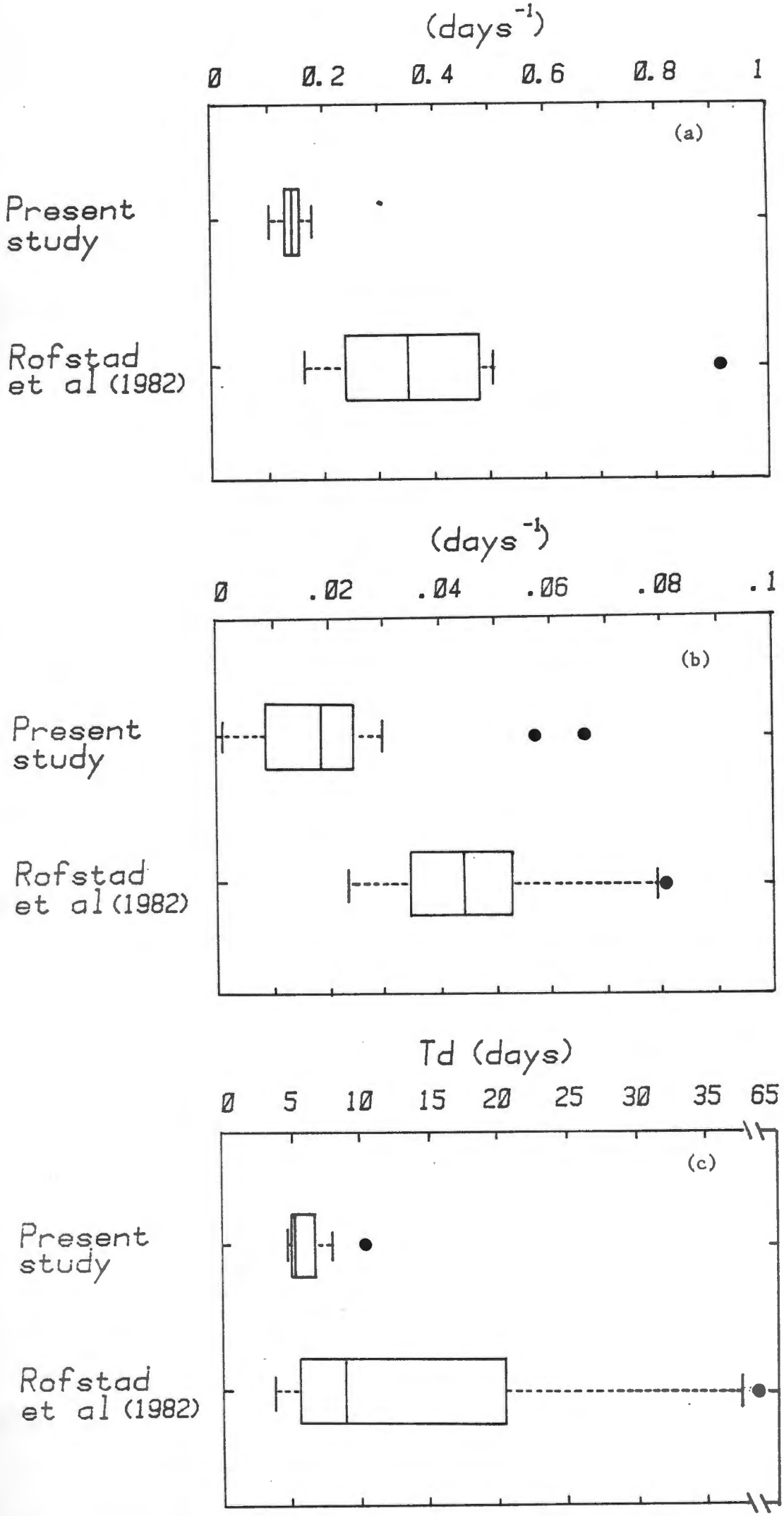
Figure 1.12

Values for growth rates of melanomas in nude mice: comparison of Gompertzian parameters calculated by data presented in this thesis with those parameters presented by Rofstad et al. 1982.

In this figure the data presented in Table 1.2 are summarized graphically and compared with those parameters of Rofstad et al. 1982 for (a) alpha (b) beta (c) $Td(200mm^3)$.

In each case the data are represented as "box and whisker" plots drawn according to the method of Tukey , Cleveland and McGill, 1985. The vertical lines inside the box indicate the 50th percentile value; the left- and right-hand ends of the box are the 25th and 75th percentiles respectively. The short vertical lines at the extremities of the dashed "whiskers" denote the "adjacent values" derived as follows: If t = 1.5x (75th percentile - 25th percentile) then the right-hand adjacent value is the largest observation that is less than or equal to the 75th percentile + t. The left-hand adjacent value is the smallest observation that is greater than or equal to the 25th percentile - t. Observations lying outside the range of the adjacent values are plotted individually as "maverick" points. Data from a sample with a normal distribution with mean values μ and variance σ^2 would give adjacent values of approximately $\mu \pm 2.67\sigma$. One would thus expect only a small fraction of the observations to fall outside the adjacent values.

Figure 1.12


(days^{-1})
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Rofstad et al (1982)
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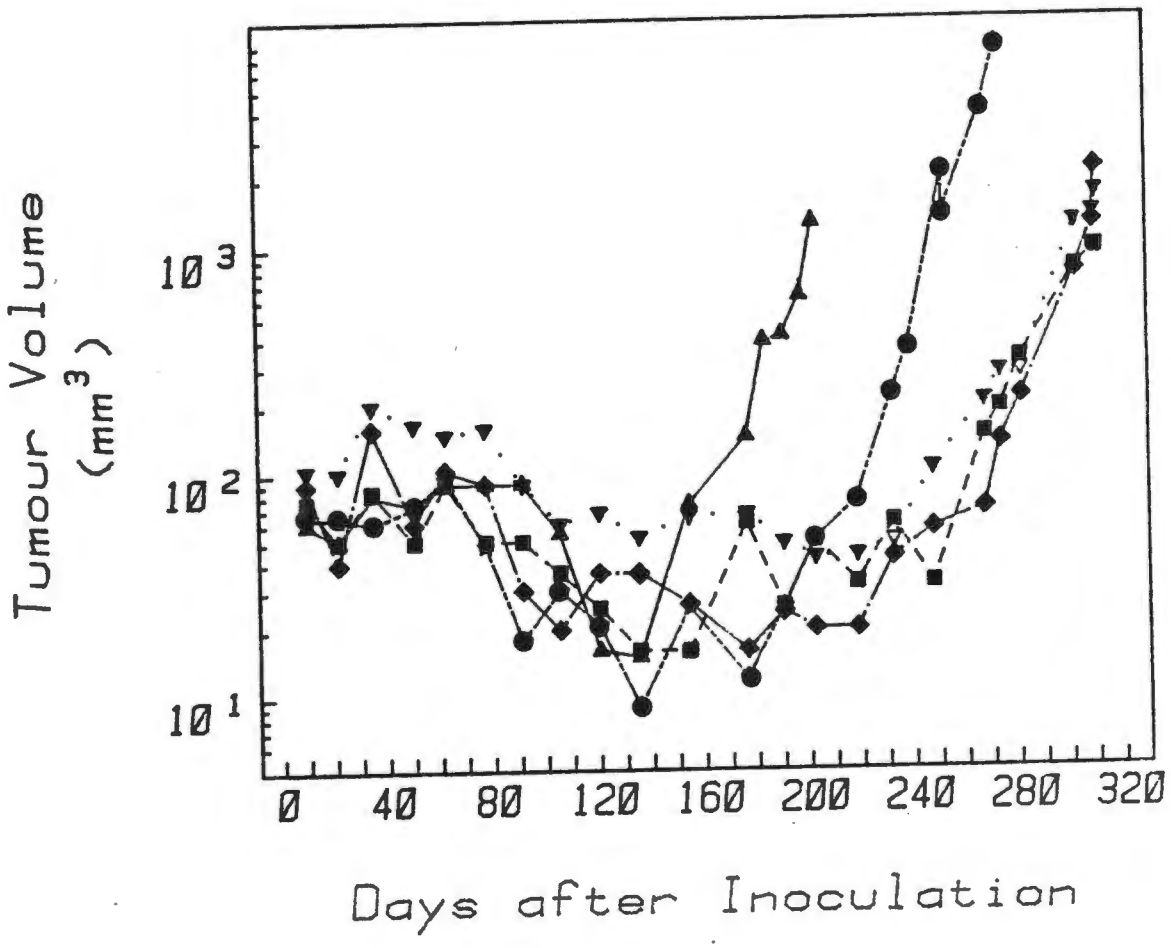


Figure 1.13

Growth of human melanomas in nude mice

Each line and set of points represents data accumulated from a single mouse. 5×10^6 UCT-Mel 7 cells (passage number 30') were inoculated subcutaneously into nude mice. After a period of early growth (approximately 80 days), growth ceased at a plateau followed by a regression in tumour size. Then, after a prolonged period of time (about 200 days) post-inoculation, the dormant tumours began to grow vigorously showing simple exponential growth kinetics.

Histological appearance of tumours

The tumours that arose from cells inoculated into the nude mice were compared, histologically, with the original material obtained from the patient. These comparisons are presented photographically in Figures 1.14. In all cases the tumour xenografts were similar, in most respects, to the original human tumour material. This was evident in the cellular morphology, the presence or absence of pigment and in the way in which the cells were organized within the tumour and disposed in relationship to each other and to the neoplastic stroma. It is of particular interest to note that, in the case of UCT-Mel 2, the tumour taken from the original had a variegate appearance with areas of intense pigmentation and other areas that were amelanotic. Exactly the same pattern was seen in tumours removed from the nude mice (Figure 1.15).

UCT-Mel 3 was a non-pigmented tumour in the patient and grew similarly in the mouse without melanin deposition. It characteristically caused extensive haemorrhage into the tumour in the mouse and for this reason appeared pigmented due to the presence of a large number of erythrocytes and degraded haemoglobin. (Figure 1.16).

The original UCT-Mel 4 tumour was deeply pigmented with strong epithelioid characteristics. When established in tissue culture the cells maintained their epithelioid morphology but lost the pigmented phenotype. The grafted tumour was non-pigmented and the histological appearance after resection was that of an undifferentiated melanoma. These appearances were similar to those of parts of the original tumour which were similarly undifferentiated.

UCT-Mel 5 was non-pigmented in both the original tumour and the xenograft and both showed a mixture of spindle-shaped and epithelioid cells. Minor differences were noted in the architecture of the original and the xenografted

FIGURE 1.14

Figure 1.14

Histological comparison of original tumours with tumours after passage in nude mice.

The complete figure displays photographs of the histological appearance of the tumours as obtained from the patient (left half of each photograph) and the corresponding tumours after passage through the nude mice (right half of the photograph). It will be noted:-

(a) UCT-Mel 1 - both tumours displayed pigmented, undifferentiated malignant melanoma, with vesicular nuclei and large nucleoli. The similarities in this case are striking.

(b) UCT-Mel 2 - the mouse tumour showed undifferentiated tumour cells with large nucleoli. Occasional pigmented cells scattered over the whole field can be noted. The parent human tumour, on the left, showed similar cells, also occasionally pigmented and lightly stained which are at present mainly in the right upper quadrant.

(c) UCT-Mel 3 - the parent tumour was an epithelioid cell malignant melanoma and the mouse tumour consisted of undifferentiated malignant melanoma. When comparing individual cells one notes that both tumours have cells with large irregular nuclei and prominent nucleoli; and that the cells on both tumours have abundant cytoplasm. The separation of the cells, more obvious in the parent tumour, may be no more than an artefact.

(d) UCT-Mel 4 - in the parent human tumour, on the left, the pigmentation is dominant whereas this feature is not illustrated in the mouse tumour. Where the parent tumour is unpigmented, as in the right lower quadrant the similarities with the mouse tumour are more evident. Both show large vesicular clear nuclei and prominent nucleoli.

(e) UCT-Mel 5 - both tumours were undifferentiated malignant melanomas, with mitoses evident centrally in the mouse tumour. The undifferentiated tumour cells with large irregular nuclei and prominent, though not really big, nucleoli are evident both in the parent tumour and the mouse tumour.

(f) UCT-Mel 7 - the parent tumour was a spindle cell type malignant melanoma and the mouse tumour was an undifferentiated malignant melanoma which tended to form spindle shaped cells. Spindling is more evident in the parent human tumour but spindle shaped cells in the mouse tumour can be seen diagonally from the lower left to the upper right quadrants. Elsewhere the mouse tumour cells are undifferentiated with some large nuclei and nucleoli, but similar cells can also be found in the parent human tumour.

The scale marker in (e) represents 20 μm. All photographs are at the same magnification.

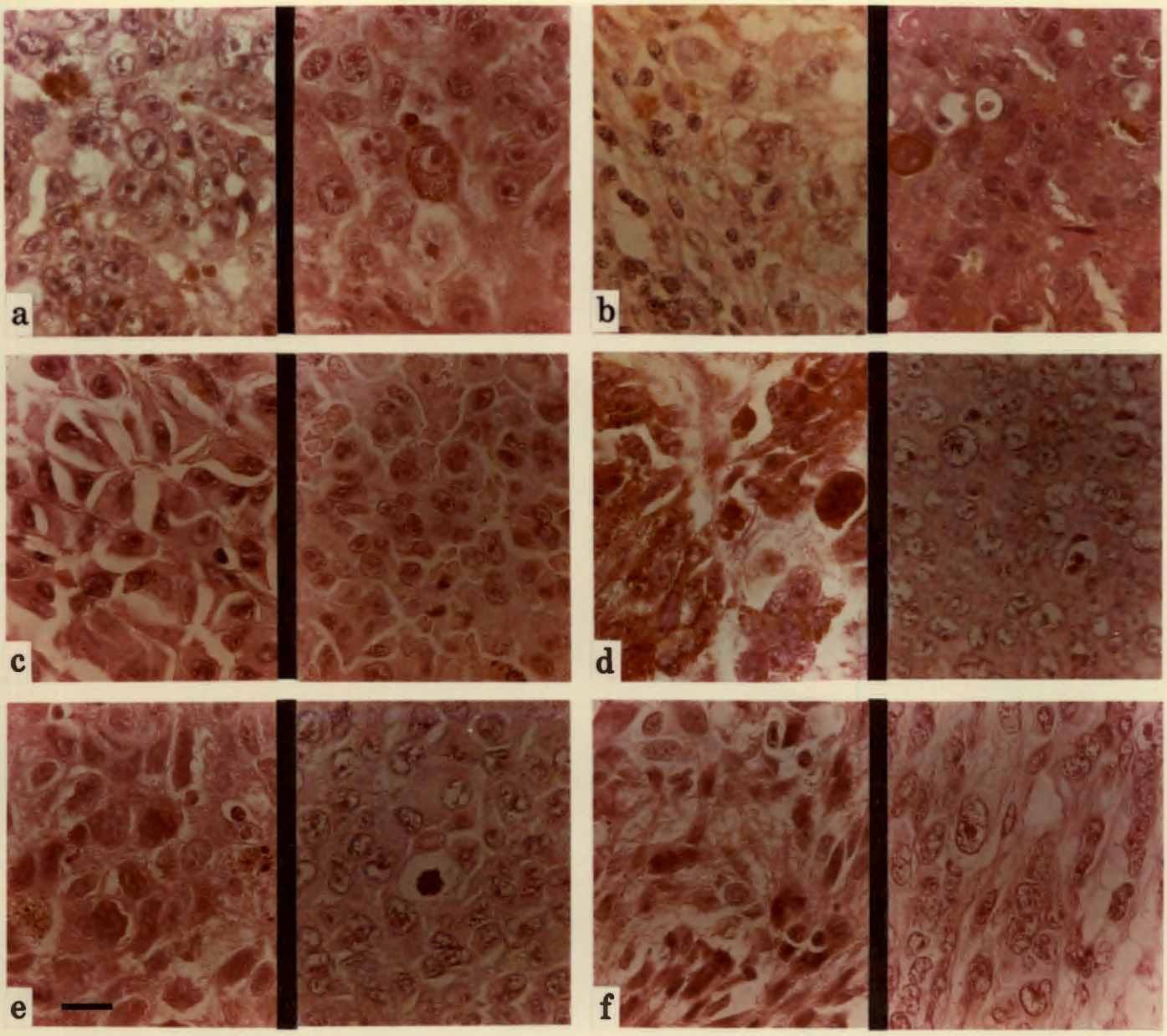


Figure 1.14

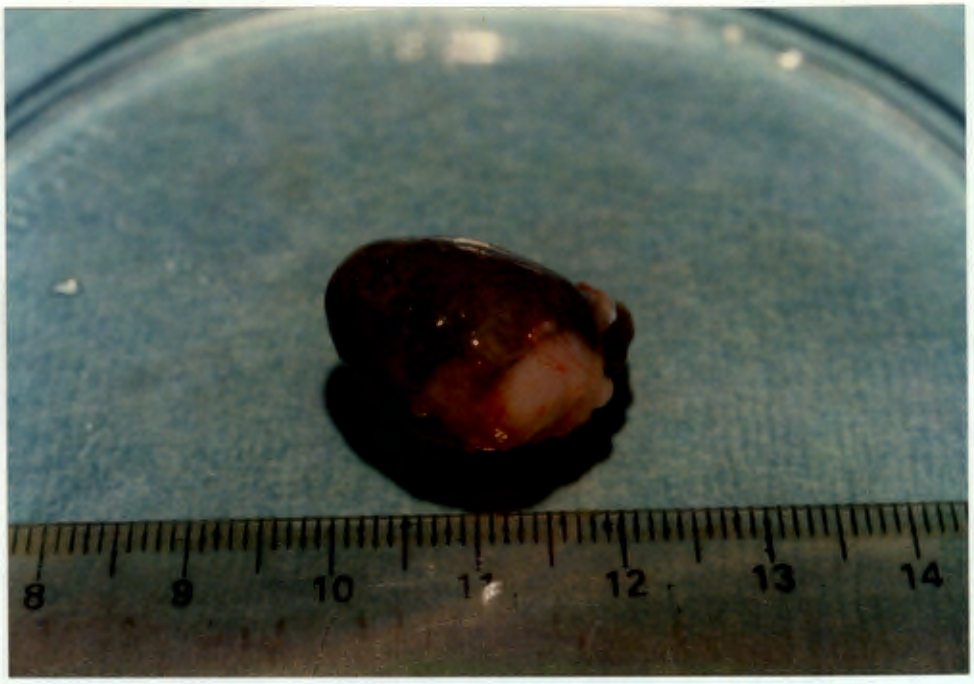


Figure 1.15

The gross appearance of UCT-Mel 2 following removal from the nude mouse

A partially pigmented tumour removed from a mouse injected with 10^6 UCT-Mel 2 cells (passage number 127'). The tumour removed from the original patient had a variegate appearance with areas of intensive pigmentation and other areas that were amelanotic. Note that exactly the same pattern is evident in the tumour removed from the nude mice.

Scale marker: each small division corresponds to 1mm.

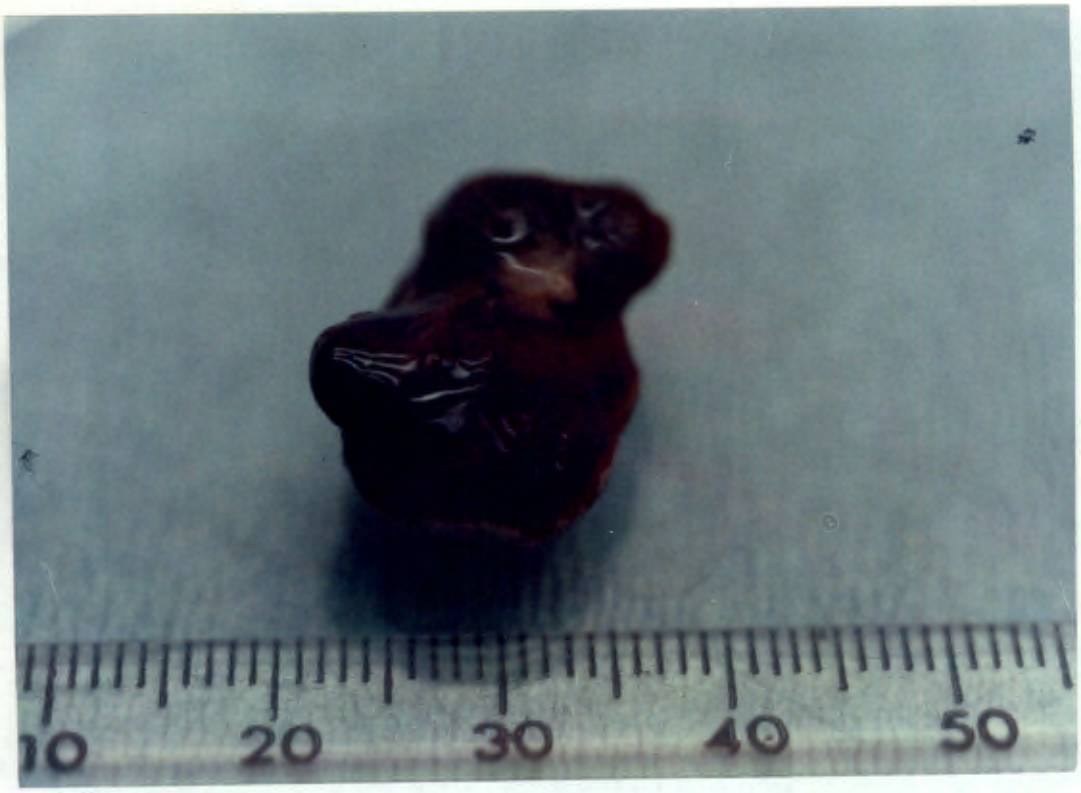


Figure 1.16.

The gross appearance of UCT-Mel 3 following removal from the nude mouse.

An apparently pigmented tumour removed from a mouse injected with 5×10^6 UCT-Mel 3 cells (passage number 69'). Histological examination of the tumour showed that the dark colour was due to blood.

Scale marker: each small division corresponds to 1mm.

tumour in that "nests" of epithelioid cells surrounded by spindle type cells that were apparent in the original tumour were not seen in the nude mouse.

UCT-Mel 7 grew as a mixture of both spindle and epithelioid cells in both the xenograft and in the original tumour. The striking feature of this particular tumour, particularly when it reached the exponential plateau phase of its growth curve, was the intense infiltration of the tumour with murine fibroblasts, macrophages and connective tissue (Figure 1.17).

Tumorigenicity as a function of inoculum size

Tumorigenicity of the inoculum in female mice was studied as a function of the inoculum size with cells from three of the lines - UCT-Mel 2, UCT-Mel 3 and UCT-Mel 7. The results showed that 10^6 or more cells were consistently tumorigenic in all of the animals; 10^5 cells gave rise to tumours in 0-40% of the mice inoculated, with variability between the cell lines studied and between different experiments and 10^4 cells were uniformly non-tumorigenic. (Table 1.5)

Growth of tumours in male and female mice

In one case (UCT-Mel 2) the growth of melanoma cells in male and female mice was compared and a striking difference was noted, between the two sexes, in the size of the inocula required to produce tumours. Inocula of 10^5 cells regularly gave rise to tumours in male mice whereas 10^5 cells were consistently non-tumourigenic in females. Once established the tumours grew with the same kinetics in males and females. Data are presented in Figure 1.18.

Metastatic spread

Provided the primary tumours that arose at the site of inoculation were excised before they attained such a size that ulceration, haemorrhage or other complications led to the death of the hosts, all of the lines that formed

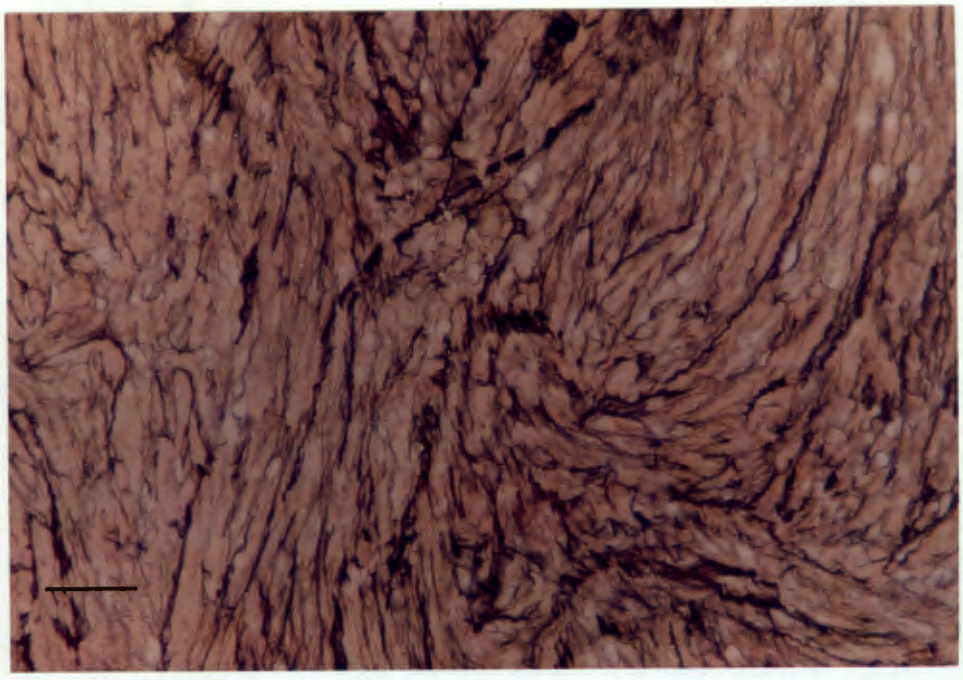


Figure 1.17

UCT-Mel 7 removed from the nude mouse and stained for reticulin

A histological section of UCT-Mel 7 was stained for reticulin (Gordon and Sweet, 1936). The tumour shows abundant reticulin arranged in bundles or around cells. UCT-Mel 7 tumours cultured *in vitro* were always heavily contaminated with mouse fibroblasts and macrophages.

The scale marker represents 100 μm.

Table 1.5

TUMORIGENICITY AS A FUNCTION OF INOCULUM SIZE IN FEMALE MICE

Cell Line	Inoculum size (cells)	Mice with tumour/ Mice inoculated
UCT-Mel 2	10^4	0/5
	10^5	0/5
	10^6	15/15
UCT-Mel 3	10^4	0/5
	10^5	0/5
	10^6	5/5
UCT-Mel 7	10^4	0/5
	10^5	2/5
	10^6	5/5

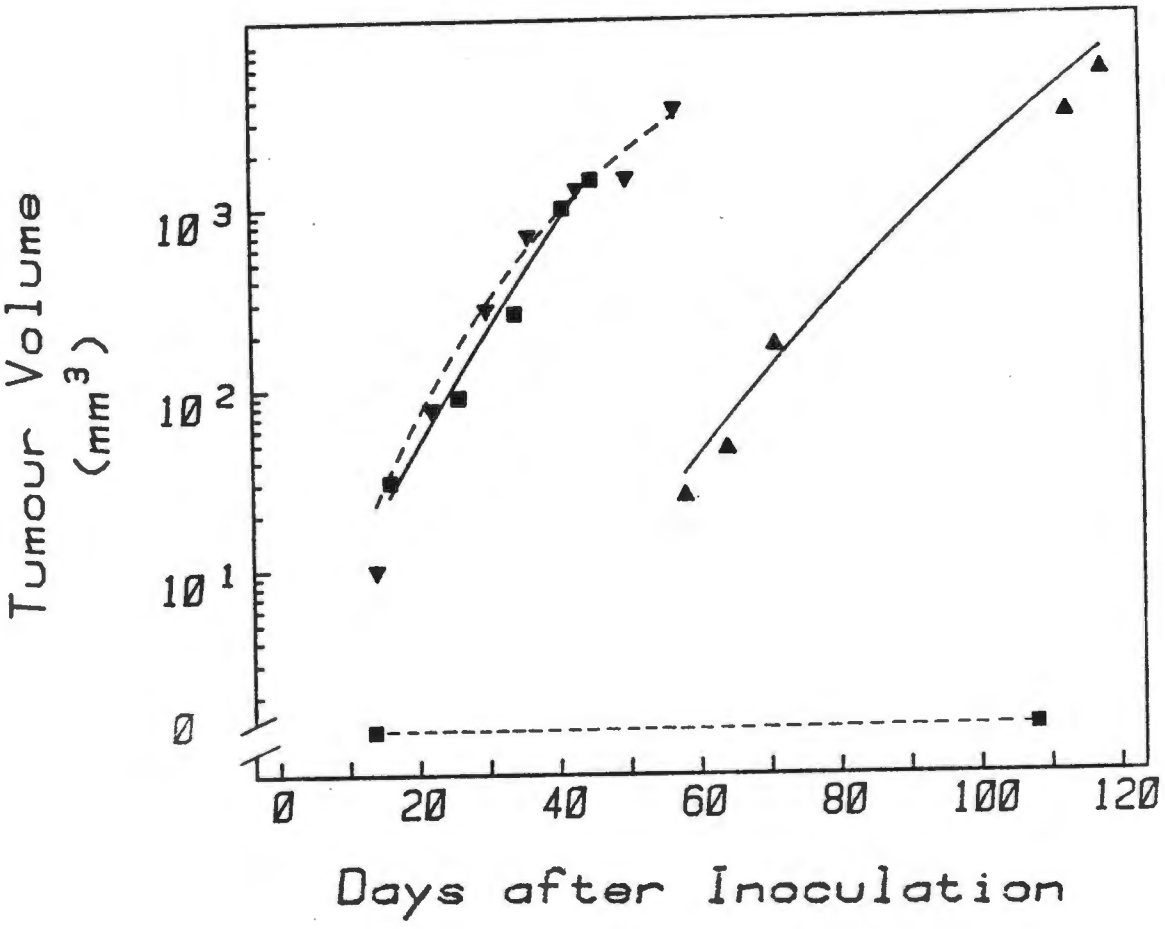


Figure 1.18

The effect of sex on the growth of human melanomas in nude mice.

Male and female mice were inoculated subcutaneously with 10^5 or 10^6 melanoma cells on Day 0 and the tumour volume determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 2 mice in the case of 10^5 cells in male mice or 5 mice in the case of 10^6 cells in male and female mice.

It will be noted:-

(a) that 10^5 melanoma cells were consistently non tumorigenic, when inoculated into female mice, whereas in male mice they regularly gave rise to tumours;

(b) that the larger the inoculum size the shorter the delay time.

The lines are as follows:- 10^5 UCT-Mel 2 cells/male (▲——▲); 10^5 UCT-Mel 2 cells/female (■---■); 10^6 UCT-Mel 2 cells/ male (▼---▼); 10^6 UCT-Mel 2 cells/female (■——■).

tumours, with the exception of UCT-Mel 7, metastasized to a greater or lesser extent.

The presence or absence of metastases was determined by detailed autopsy of the animals that were killed, or that died spontaneously after the excision of the primary tumour. Killing the animals too soon (i.e. before metastatic foci had grown to a readily detectable size) would have resulted in an underestimate of the metastatic frequency. Leaving them too long before scoring, on the other hand, would have caused logistic difficulties in housing and maintaining the mice for long periods. It was therefore necessary to define criteria for deciding when to terminate the experiment on each mouse.

Taking the diameter of a melanoma cell as 20 microns, its volume would be 4.2×10^{-6} mm^3. A spherical tumour comprising a million such cells would thus have a volume of 4.2mm^3, or a diameter of 2mm. A metastatic deposit of this size should be readily visible.

Assuming that small tumours grow exponentially <u>in vivo</u> with a doubling time of 4 days (cf. Table 1.3) it should have taken approximately 80 days for a single cell to give rise to a visible deposit. Observing the mice for at least 120 days after excision of the primary tumour should have provided a reasonable chance of detecting any metastases that were going to develop. In some cases this criterion was used and mice were not autopsied for at least four months after excision of the tumour.

In most cases, however, I relied upon the useful observation that the presence of metastases in otherwise apparently healthy animals could usually be detected by following the body weight with time. Excision of the primary tumour usually resulted in an increase in body mass to a plateau which persisted until metastases developed when a decline in weight was observed (Figure 1.19).

The cell lines differed remarkably in the frequency with which they formed metastatic tumours (Table 1.6), ranging from 6% in the case of UCT-Mel

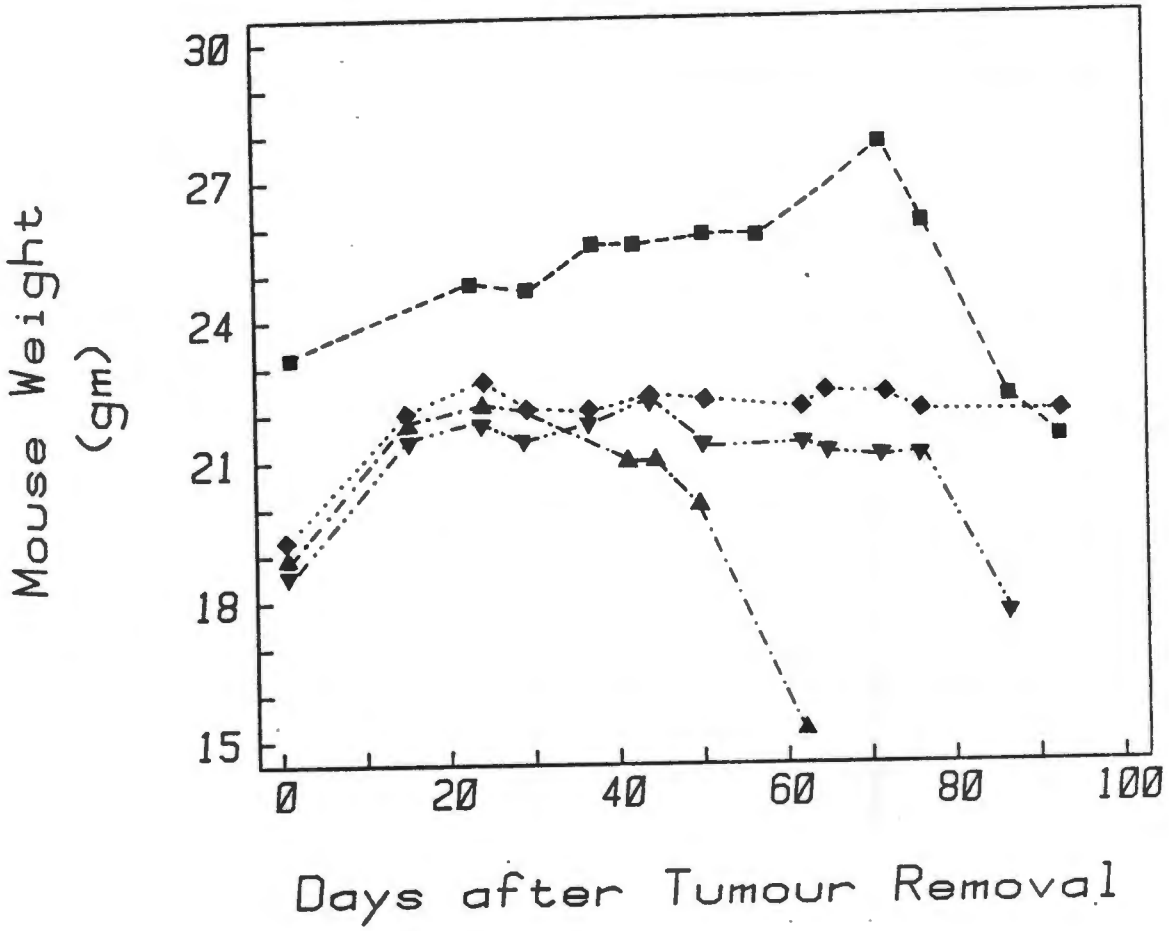


Figure 1.19 .

Decline in body weight after removal of the primary tumour.

Each line and set of points represents data accumulated from a single mouse. Mice were inoculated subcutaneously with 10^6 or $5x10^6$ UCT-Mel 3 cells (passage number 69'). Tumours were removed on Day 0, when they reached approximately the size of 2000-3000mm^3. Note that the excision of the primary tumour usually resulted in an increase in body mass to a plateau after which a decline in weight was observed which coincided with the presence of metastatic disease.

Table 1.6

METASTASES IN NUDE MICE

Cell line	No. of mice	Metastases		Maximum period of observation (days)	Site of metastases
		No.	%		
UCT-Mel 1	17	1	6	260	Lung
UCT-Mel 2	26	3	12	380	Lung
UCT-Mel 3	35	35	100	190	Lung, gonads, kidney, liver, intestine
UCT-Mel 4	10	1	10	270	Lung
UCT-Mel 5	22	3	14	313	Lung
UCT-Mel 7	10	0	0	370	-

1 to 100% with tumours from UCT-Mel 3. Tumours arising from the interscapular primary mass were deposited in a number of sites including the abdominal cavity, the gonads, kidney, lungs and liver.

Metastases detected at autopsy were re-implanted into mice and repeatedly passaged as subcutaneous tumours *in vivo*. Their *in vivo* growth kinetics were compared with those of the parent primary tumour from which they were derived. No significant differences in growth characteristics were observed (Table 1.7 and Figs. 1.20 to 1.24 and Appendix Table A.8).

DISCUSSION

Growth kinetics

In most respects the observations that I have made on the growth of tumours that develop from human melanoma cells inoculated into nude mice are in accord with observations made by others (Rofstad et al., 1982; Fodstad et al., 1980; Giovanella et al., 1972, 1973, 1984; Fogh et al., 1977; Aubert et al., 1976, 1980).

Following a period of latency that was inversely related to the number of cells implanted, tumours developed in most cases. These grew progressively with a decelerating growth rate.

Several mathematical models have been proposed to describe growth curves (Peters, 1983; Defares and Snedden, 1964). Four of the most popular are the Gompertzian, the Logistic, the von Bertalanffy and the "mononuclear".

All require, in one form or another, three constants:

(i) The minimum size to which the equation applies (Vo), or a maximum size to which the volume of the function tends (Vmax),

(ii) An experimental rate constant of growth (alpha in our case)

(iii) A second rate constant that modifies the fractional change in growth rate as a function of size. In our case this is represented by beta.

Usually these alternative models all give an equally good fit to the

Table 1.7

GROWTH IN VIVO OF PARENT PRIMARY TUMOUR AND METASTATIC DEPOSITS

Cell line	Passage in vivo (1)	Td(days) (V=200mm3)
UCT-Mel 1		
Parent tumour	-	5.3
Lung metastases	4	5.6
UCT-Mel 2		
Parent tumour	-	6.1
Lung metastases	6	4.8
UCT-Mel 3		
Parent tumour	-	5.4
Lung metastases	11	6.0
UCT-Mel 4		
Parent tumour	-	4.9
Lung metastases	1	3.2
UCT-Mel 5		
Parent tumour	-	8.0
Lung metastases	5	9.6

(1) Metastatic deposits were removed from mice inoculated with cells cultured *in vitro* and these were re-implanted subcutaneously into fresh recipients. The tumours that resulted were repeatedly passaged *in vivo* and their growth characteristics measured after the indicated number of such passage.

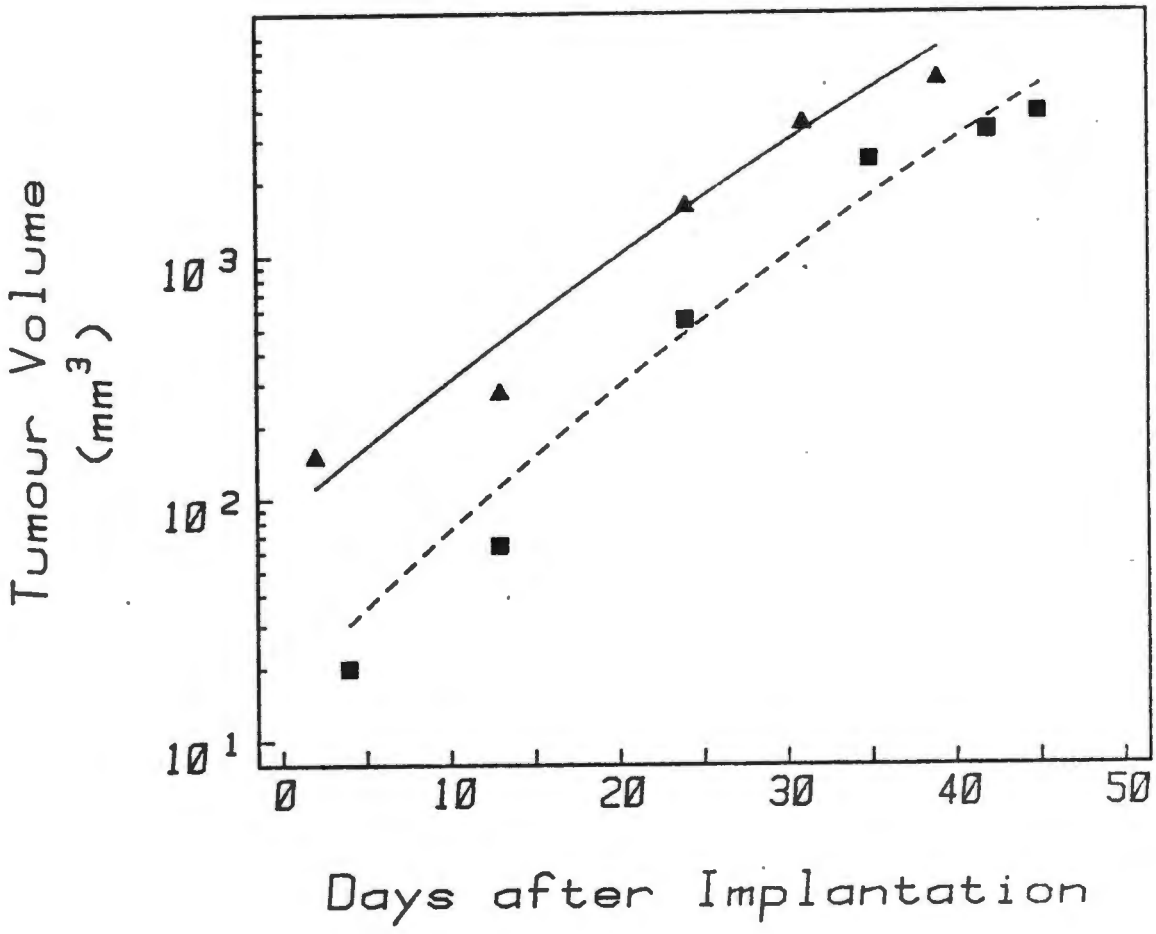


Figure 1.20

Growth in vivo of UCT-Mel 1 parent primary tumour and metastatic deposits removed from the lung.[6]

Mice were inoculated subcutaneously with 5x10 melanoma cells (in vitro passage number 65') or metastatic explants of in vivo passage 4, on Day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice in the case of the parent tumour or for 4 mice in the case of the metastatic explants. Note that no significant differences in growth characteristics were observed.

The tumours illustrated are as follows: UCT-Mel 1/parent tumour (■----■); UCT-Mel 1/metastatic deposits (▲——▲).

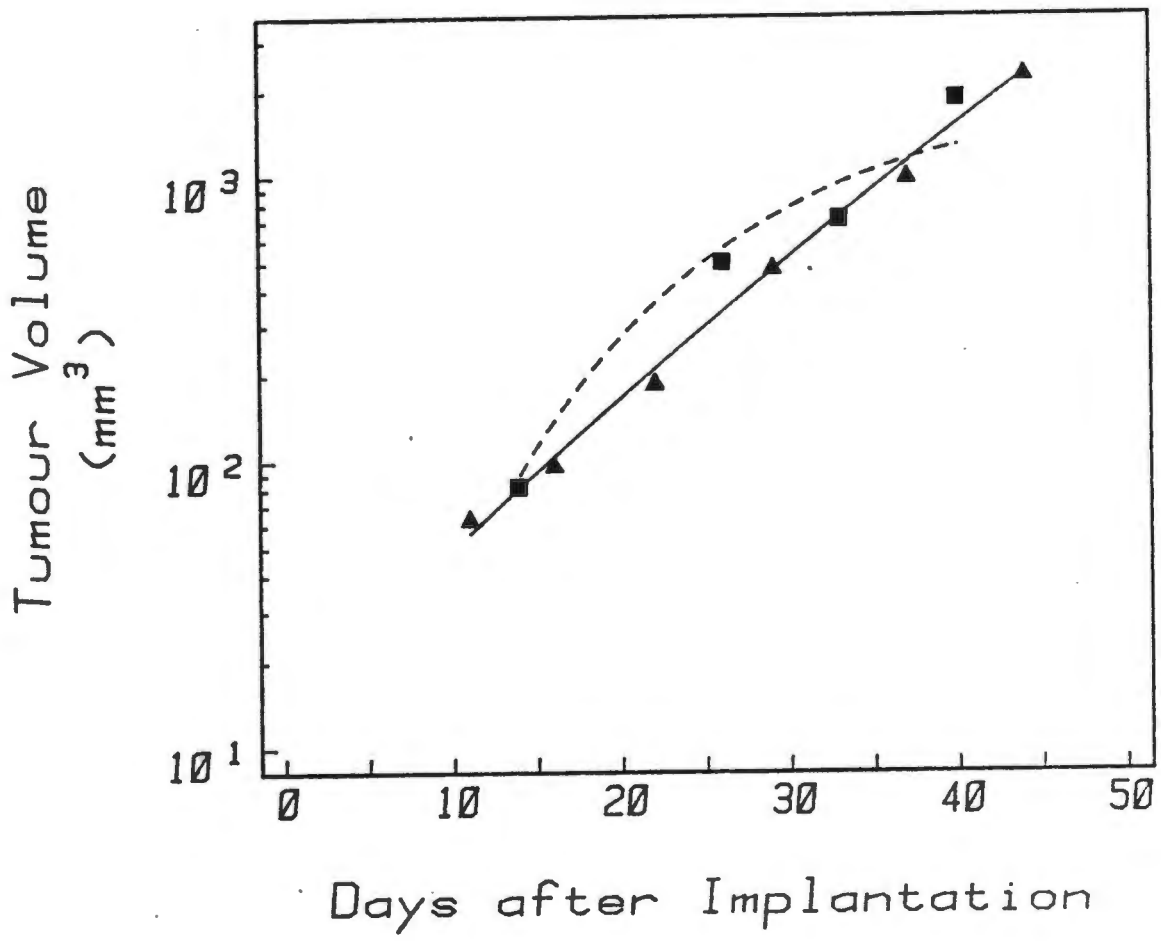


Figure 1.24

Growth in vivo of UCT-Mel 2 parent primary tumour and metastatic deposits removed from the lung.[6]

Mice were inoculated subcutaneously with 5x10 melanoma cells (in vitro passage number 124') or metastatic explants of in vivo passage 6, on Day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice in the case of parent tumours or for 2 mice in the case of metastatic deposits. Note that no significant differences in growth characteristics were observed.

The tumours illustrated are as follows: UCT-Mel 2/parent tumour (▲——▲); UCT-Mel 2/metastatic deposits (■----■).

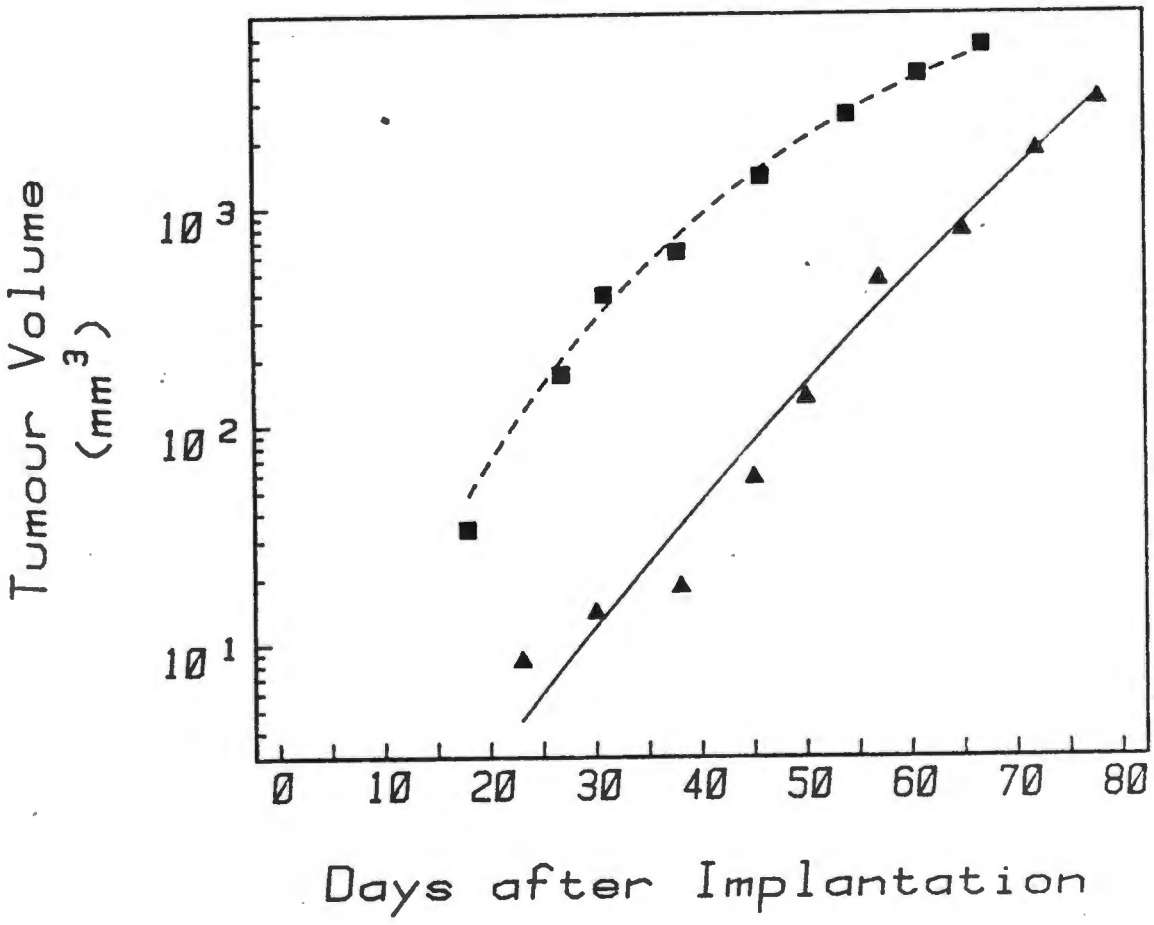


Figure 1.22

Growth in vivo of UCT-Mel 3 parent primary tumour and metastatic deposits removed from the lung.

Mice were inoculated subcutaneously with 2×10^6 cells obtained from a metastatic deposit from the lung which had been cultured *in vitro* for 11' passages or 5×10^6 UCT-Mel 3 melanoma cells (*in vitro* passage number 69') on day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice in the case of the parent tumour or for 4 mice in the case of the metastatic deposits. Note that no significant differences in growth characteristics were observed.

The tumours illustrated are as follows: UCT-Mel 3/parent tumour (■----■); UCT-Mel 3/metastatic deposits (▲——▲).

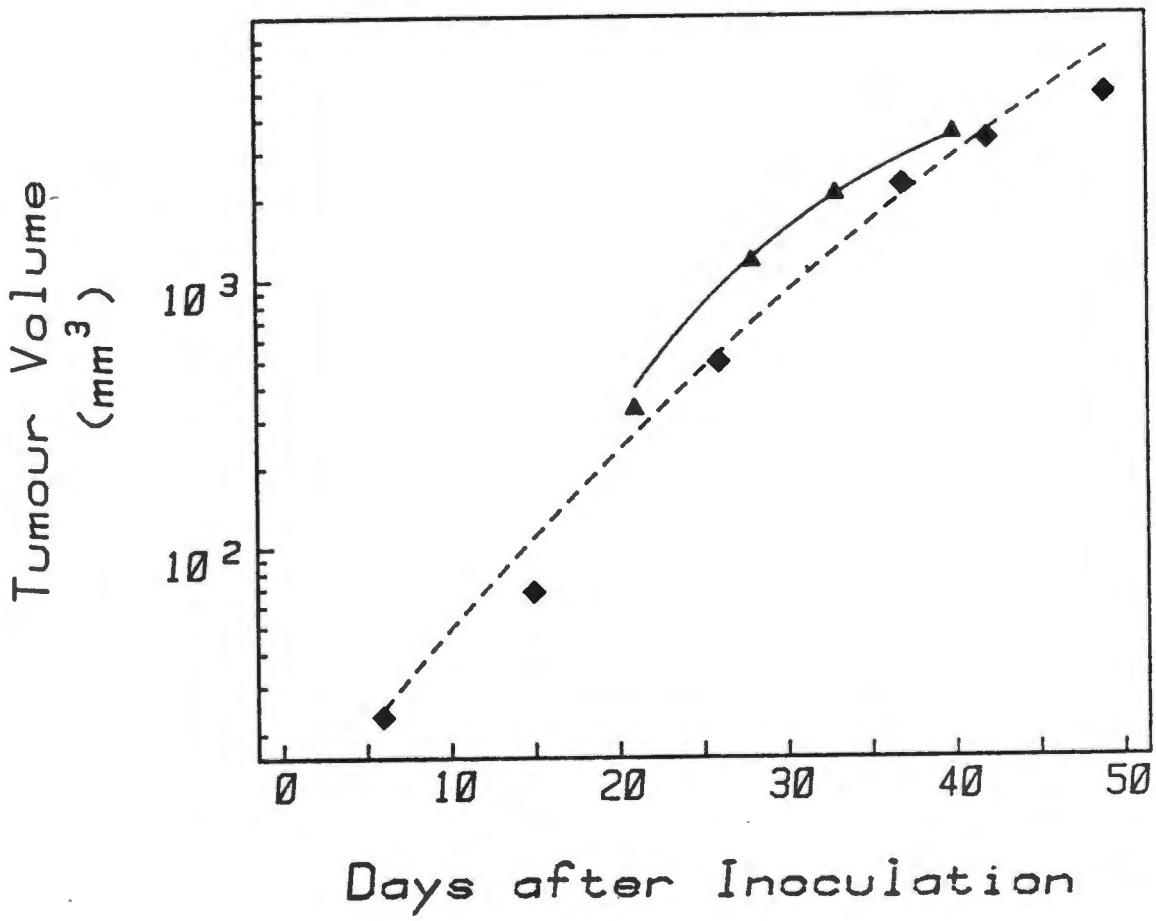


Figure 1.23

Growth in vivo of UCT-Mel 4 parent primary tumour and metastatic deposits removed from the lung.[6]

Mice were inoculated subcutaneously with 5x10 melanoma cells (in vitro passage number 20') or metastatic explants of in vivo passage 1, on Day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice in the case of the parent tumour and 2 mice in the case of the metastatic deposits. Note that no significant differences in growth characteristics were observed.

The tumours illustrated are as follows: UCT-Mel 4/parent tumour (◆--◆); UCT-Mel 4/metastatic deposits (▲——▲).

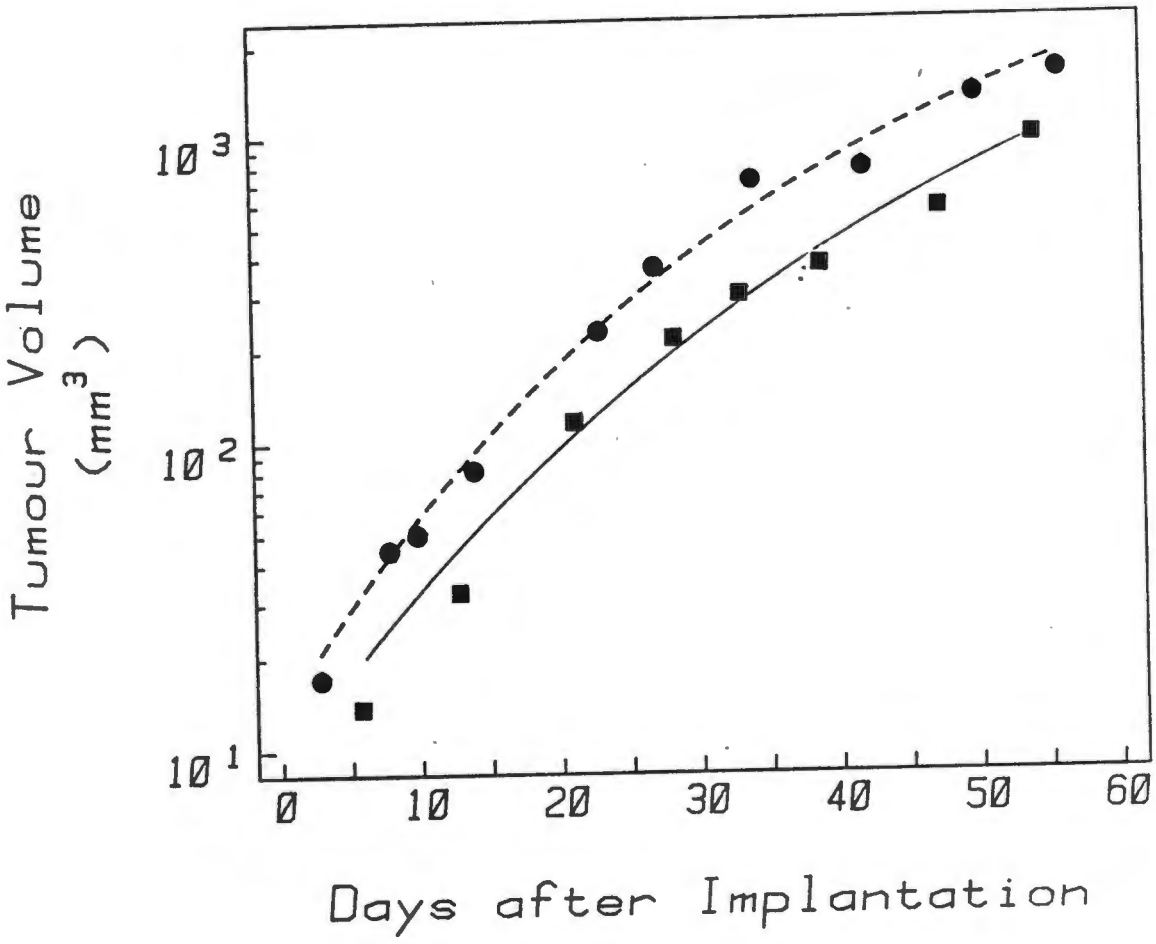


Figure 1.24

Growth in vivo of UCT-Mel 5 parent primary tumour and metastatic deposits removed from the lung.

Mice were inoculated subcutaneously with 5×10^6 (*in vitro* passage number 27') melanoma cells or metastatic explants of *in vivo* passage 5 on day 0 and the tumour volumes determined at the indicated times. The points on the graph were calculated using the Gompertz function and they represent the mean values for 5 mice. Note that no significant differences in growth characteristics were observed.

The tumours illustrated are as follows: UCT-Mel 5/parent tumour (●----●); UCT-Mel 5/metastatic deposits (■——■).

experimental data (Ricklefs, 1967) and I know of no sound *a priori* reason for choosing one to the other.

In two papers published 20 years ago, Laird (1964; 1965) drew attention of oncologists to the Gompertz equation and since, I suspect, there has been no compelling reason to seek more accurate models, the Gompertzian function has stayed with us.

Although useful devices for describing the dynamics of tumour growth, these mathematical models are not generally used to give quantitative expression to well-defined underlying biological processes and one does not find any tendency in the literature to sustain rationale for their use.

This, in spite of the fact that it is reasonable, on first principles, to think of tumour growth as the result of two processes: (a) an exponential increase in tumour size that will cause the tumour to enlarge at a rate that is proportional to its size and (b) modifying phenomena such as limitation of blood supply or diffusion of nutrients, that exert a negative effect upon growth rate that is also dependent on size i.e. the larger the tumour the more rapidly its growth rate will decelerate. The magnitudes of alpha and beta give some idea of the relative contribution of these two processes but it should be remembered that models that involve exponential parameters can be fitted to many relationships and when, as in the case of the Gompertzian curves, double exponentials are involved the models become even more suspect. As Gauss is reputed to have said, "give me three parameters and I'll draw you an elephant; give me a fourth and I'll make it wag its tail!". The value of *in vivo* growth measurement as a function of time is greater if these are considered in conjunction with simultaneous cytokinetic data obtained with *in vivo* cell labelling techniques and determination, by flow cytometry, of the fraction of cells in different stages of the cell cycle. This subject has been extensively reviewed by Steel (1977). The value of this combined experimental approach to the analysis of the kinetics of tumour growth is well

illustrated by the papers of Spang-Thomsen and Vindelov,(1984), Shirakawa et al., (1970) and Rofstad et al., (1982).

The empirical fact of the matter is that my data were well fitted by a Gompertzian curve and the parameters obtained could be used to calculate theoretical delay times and instantaneous doubling times at a given tumour volume. They could also be used to compare the results that I obtained with those of others who have studied mice bearing human melanoma xenografts.

The kinetic data that I have accumulated warrant comment in the following itemized respects:

(i) The Gompertzian parameters that I calculated were very similar to those reported by others (Spang-Thomsen et al., 1980; Rofstad et al., 1982). My data showed slightly less scatter than those of Rofstad et al., (1982) (Figure 1.13), due, in all probability, to differences in the strain of nu/nu mice used, the tumours studied and techniques used to measure tumour volume.

(ii) Volume doubling times in vivo (approximately 4-5 days) in the mouse are longer than the cell cycle time (30-50 hr) as measured in vitro or in vivo by DNA cell labelling techniques (Quastler and Sherman, 1959). There is general agreement that, while this difference may be attributable in part, to a diminished growth fraction in vivo, it is due to a greater extent, to cell loss from metastasis, exfoliation or cell death (Frindel et al.,1967,1968; Shirakawa et al., 1970; Spang-Thomsen and Vindelov, 1984; Rofstad et al., 1982; Steel,1977). Furthermore, the retardation in growth rates seen as tumours enlarge is similarly due to an increase in the cell-loss factor and not due to a decrease in growth fraction (Frindel et al., 1967; Steel, 1967; Tannock, 1969).

(iii) Few reports are available on melanoma-volume doubling times in vivo in man. Nathenson et al (1967) have reported values of 2-16 weeks with a median of 6 weeks for metastases in the lung of patients x-rayed sequentially. A similar study reported by Rampen (1982) recorded doubling times ranging from

2-15 weeks (median approximately 6 weeks). These data and common clinic experience, tell us that tumours grow much more rapidly in nude mice than they do in man. This may well be due to the fact that the growth fraction of melanomas in the human host is 20% - 25% (Shirakawa et al., 1970) when xenografted melanomas in mice show growth fractions of 60-80% (Spang-Thomsen and Vindelov, 1984) although Rofstad et al (1982) found that only 5-25% of xenografted melanoma cells were in the S phase of the cell cycle.

(iv) The time taken to reach a theoretical volume of $100mm^3$ provided a reasonable estimate of tumour latency that showed the expected inverse correlation with inoculum size. Since, all other things being equal, an increase in inoculum size should mean an increase in the number of clonogenic cells injected, the Tdel(100) might be used as a measure of the effect of hormones, drugs or other manipulations on the number of clonogenic cells in inocula of constant size. I have used this as one of the parameters to study the effect of hormones on in vivo tumour growth (Chapter 3).

In summary, the experiments that I report in this section have shown that it is possible to evaluate growth rates for human melanomas xenografted into nude mice and that, within limits, these data are consistent for any given line and amenable to measurements with sufficient precision to permit their use in experiments designed to identify factors that modulate tumour growth. It should, however, be emphasized that volume growth in vivo in the nude mouse is the complex result of several factors that involve host factors, the cells themselves and interactions between the two. Furthermore, there are obvious differences between growth rates in man and the mouse that indicate the need for caution in extrapolating conclusions from one species to the other, despite the great similarities that exist between the histological appearances of the tumour in the original human host and in the xenograft (Fig.1.14).

Two of the cell lines behaved atypically when inoculated into nude mice. The first, UCT-Mel 6, did not form tumours despite all efforts to induce

growth by increasing the inoculum size or by co-inoculation of the tumour cells with fibroblasts. It is of interest to note that these cells, although from a tumour that was confidently diagnosed as a malignant melanoma, were unlike other melanoma cells in other respects. When studied *in vitro* they failed to grow in an anchorage-independent manner and they synthesized plasminogen activators of the urokinase type (whereas other melanomas all synthesized tissue plasminogen activator) and they lacked the melanoma-specific M19 antigen (Hoal-van Helden et al., 1986). I have no good explanation for the lack of tumorigenicity shown by these cells. It is a question that clearly deserves further study for the light that it may shed on phenotypic variation in melanoma cells and the way that these influence host:tumour cell interactions.

The second anomalous cell line, UCT-Mel 7, behaved consistently in the most interesting manner. After a period of rapid initial volume increase the tumour size remained constant and then regressed to become barely palpable. Finally, the fourth phase was seen in which the tumour abruptly recurred and grew with exponential vigour. The tumour was also of interest for the remarkable desmoplastic response that it elicited. More detailed studies with this cell line are reported in Chapter 5.

Tumorigenicity

As I have already indicated, all of the cell lines, with the exception of UCT-Mel 6, gave rise to tumours. I should like, at this point, to emphasize the fact that tumorigenicity in the nude mouse, although generally regarded as a qualitative attribute that characterizes malignant cells, has a quantitative component that may, in fact, be more informative. I refer to the fact that the cells from UCT-Mel 2, 3 and 7 showed a clear relationship between tumorigencity and the size of the inoculum. This similar relationship has been well-documented by others for melanomas and other tumours (Kindred and

Wechsler,1978; Stiles et al.,1976; Peters and Hewitt,1974; Woodruff and Dunbar,1974; Denys,1972; Vladimer et al., 1978) although relatively few (Porter et al.,1973; Hewitt and Wilson, 1959; Hewitt et al., 1967) have published systematic studies of this phenomenon.

It is assumed that an inoculum will only cause a tumour if it contains one or more clonogenic cells. If there are an average of m clonogenic cells per inoculum, the probability of a failure to produce a tumour is given by the Poisson distribution as $p = e^{-m}$. Finney, (1964) has developed a useful bio-assay, based on this relationship, for determining the TD50 (i.e. the inoculum size that will give rise to a tumour in 50% of recipients) and for detecting anomalous transplantation kinetics where "single clonogenic cell" kinetics do not apply.

Where experiments aim to quantitate the effects of various manipulations on the "tumorigenicity" of a cell line, it would clearly be desirable if precise methods for estimating tumorigenicity could be devised based upon a TD50 rather than upon a binary "yes/no" appraisal of whether or not the cells could be induced to grow. I take this matter up again in Chapter 2.

It is also apparent, from data that I present in Fig.1.18, that the likelihood of a tumour developing from a marginally (or a sub-) tumorigenic inoculum may be related to the sex of the recipient. Relatively few studies have produced definitive experimental evidence showing that human melanomas are hormonally dependent tumours. I consider this question in Chapter 3.

CHAPTER 2

CHAPTER 2

THE EFFECT OF CO-INOCULATED CELLS ON TUMORIGENICITY OF HUMAN MELANOMA CELLS IN THE ATHYMIC MICE.

In 1956 Révész made the interesting observation that the growth of an experimental transplanted tumour was greatly enhanced by adding lethally irradiated tumour cells to the viable inoculum. This was manifest as an earlier and higher incidence of progressively growing tumours and a decreased survival time of the tumour-bearing host. Two years later he published a second paper (Révész, 1958) in wich he reported the results of a remarkably scholarly and systematic experimental analysis of the phenomenon. He showed that inocula of tumour cells that would ordinarily not have been tumorigenic, by virtue of the low number of cells that they contained or by virtue of a host environment that was not propitious, could be induced to form tumours if co-injected with normal or irradiated cells that were not tumorigenic in their own right.

His inocula thus comprised two fractions: a population of potentially tumorigenic cells and a complementing cell fraction. No enhancement of potentially tumorigenic cell growth was seen if the complementing cells were deposited at a different anatomical site or killed by heat treatment. There was thus a need for intimate, local contact between the potentially tumorigenic cells and a complementing cell fraction that was viable in the metabolic sense if not in the proliferative sense.

The effect did not seem to be due to the induction of local inflammatory response by the complementing cell fraction nor did prior local irradiation of the transplant bed cause enhancement. Révész thus concluded that the complementing cells elaborated "growth factors" analagous to those that had been described by Sanford et al., 1948, in "conditioned medium" and that would promote the growth of single cells in culture.

At the time at which Révész made his original observations it was not common practice to apply Poisson statistics to the analysis of transplant data. He did not, therefore, plan his experiments in such a way that it was possible to draw a clear distinction between an effect of complementing cells on the growth rate of the potentially tumorigenic cell fraction or on the number of clonogenic cells that it contained. In 1973 Hewitt, Blake and Porter showed, with a number of syngeneic tumours, that the Révész effect was due to an increase in the proportion of potentially tumorigenic cells that contributed to the initiation of tumour growth. Whereas as 6900 cells was required for 50% takes (TD50), the addition of 10^6 complementing cells reduced the TD50 to 4 cells with the retention of the Poisson relationship.

I found the Révész effect interesting for a number of reasons.

In the first place it is well known that human tumours will only grow in the nude mouse if a minimum number of cells is transplanted. It would seem appropriate to ask if this requirement for a critical inoculum size means that only a small fraction of the tumour cell population is potentially clonogenic or whether a Révész phenomenon operates in the sense that the bulk of a successful inoculum comprises cells that function as complementing cells.

Secondly, most work on the Révész effect to date has been done with syngeneic systems. When Hewitt et al., (1973) attempted to use allogeneic potentially tumorigenic cell and complementing cell combinations they obtained highly variable results and they were unable to show enhancement of growth if the potentially tumorigenic cells and the host were histo-incompatible. The nude mouse, by providing an immunologically bland environment, should allow experiments designed to establish the extent to which potentially tumorigenic cell:complementing cell relatedness is required.

Thirdly, I thought it should be possible to use the nude mouse to develop assay systems for quantitating complementing cell function and so to contribute methods for the study of intercellular interactions that influence

expression of the neoplastic phenotype in man.

Finally, several major reports on the use of nude mice for the study of human tumours have recently appeared (Baldwin and Pimm, 1980; Bellet et al., 1979; Carrel et al., 1976; Dexter et al., 1982; Fogh et al., 1977; Fodstad et al., 1980; Giovannella et al., 1974a;b; 1978; 1983; 1984; Giovanella and Fogh, 1978; Giovanella and Stehlin, 1974; Gershwin et al., 1977; Hata et al., 1978; Helson et al., 1975; Merenda et al., 1975; Reid et al., 1977; Stiles et al., 1976a;b;c; Sordat et al., 1974; 1978; Sordat and Bogenmann, 1980; Sordat and Merenda, 1977) without mention of the phenomenon and a citation index literature search back to 1974 produced only 27 papers that refer to the Révész effect and, of these only two - both from the same laboratory - (Lozzio et al., 1976a; Lozzio et al., 1976b) reported on the enhancing effect of fibroblasts on the growth of the human malignant cell line, K-562, in the nude mouse. Stiles et al. (1976c) demonstrated the Révész effect in nude mice transplanted with SVT2 cells or HeLa cells using embryo fibroblasts of murine or human origin. He did not, however, study the phenomenon systematically. If nu/nu mice are to be used to set a value on the tumorigenicity of human cells, the apparent lack of attention that has been paid to the phenomenon requires, in my opinion, rectifying.

For these reasons I embarked upon a series of experiments in which I studied the effect of co-inoculating fibroblasts and other cells on the tumorigenicity of human melanoma cells. In this chapter I report the results of these experiments which showed that the Révész effect is, indeed, demonstrable with xenotransplanted cells and that it has a number of interesting features.

MATERIALS AND METHODS

Cell lines

Three of the seven human melanoma cell lines (UCT-Mel 2, UCT-Mel 3 and UCT-Mel 6) described in the previous chapter were used for this study. Human

fibroblasts were obtained from cultures of adult skin, neonatal foreskin and skin of a female embryo; murine fibroblasts were prepared from a near-term embryo. These cells were prepared using conventional techniques (Freshney, 1983).

UCT-Br1 is a breast carcinoma cell line that was established in this laboratory from a bone metastasis from a patient with primary carcinoma of the breast. UCT-Br1 was chosen for the fact that, like UCT-Mel 6, we have not been able to induce it to form tumours in the nude mouse.

Tumour cells and fibroblasts were cultured in vitro, removed from petri dishes and inoculated alone or in combination subcutaneously in nude mice as described in detail in Chapter 1. Mice were then weighed and the tumour volume measured at weekly intervals.

RESULTS

Enhancement of melanoma cell growth by co-inoculation of fibroblasts was clearly demonstrated in the case of UCT-Mel 2 and UCT-Mel 3 (Table 2.1). Tumours did not result from inocula containing 10^5 cells from either of these two lines whereas 10^6 cells consistently gave rise to tumours. When 10^5 cells were injected with 10^6 normal adult skin fibroblasts, tumours resulted in all animals injected.

Despite large inocula of tumour cells, either alone or with fibroblasts, UCT-Mel 6 was steadfastly non-tumorigenic (Table 2.1).

Diagrams depicting tumour volume as a function of time for different inocula are shown in Figs. 2.1 to 2.5. In each case, Gompertzian curves have been fitted to the average data points for each experimental set as indicated in the figure legends. These results warrant comment in the following respects:

When 10^5 UCT-Mel 2 cells are injected alone tumours did not form; 10^6 cells caused tumours in all cases. When 10^5 were inoculated with 10^6 fibro-

Table 2.1

Tumorigenicity of human melanoma cells in nude mice

Effects of co-inoculated fibroblasts

Cell Line	No. of cells injected	Fibroblasts injected(1)	Tumours (2) (No. of takes/No. of mice)
UCT-Mel 2	10^6	-	5/5
	10^5	-	0/5
	10^5	10^6	5/5
	-	10^6	0/5
UCT-Mel 3	10^6	-	5/5
	10^5	-	0/5
	10^5	10^6	3/3
	-	10^6	0/5
UCT-Mel 6	10^6	-	0/2
	$5x10^6$	-	0/2
	10^6	10^6	0/2
	10^6	10^6	0/2

(1) Adult skin fibroblasts were mixed with the melanoma cells or diluted in medium to give the stated inocula in 0.1ml volumes.

(2) Mice were observed for a minimum period of 240 days before being scored negative for tumour growth.

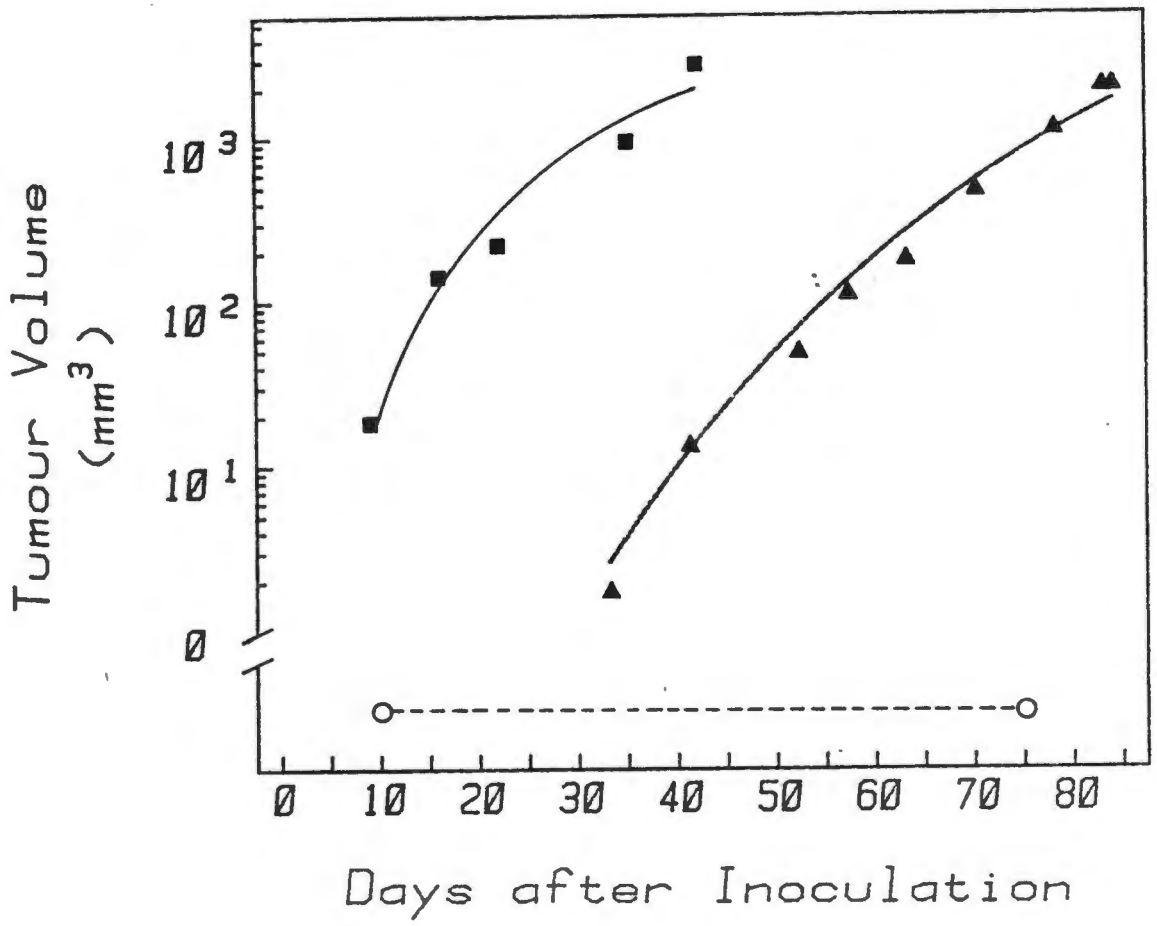


Figure 2.1

Tumour volume as a function of time for different inocula

Mice were inoculated subcutaneously with 10^5 or 10^6 UCT-Mel 2 melanoma cells, either alone or mixed with 10^6 viable skin fibroblasts. The points on the graph were calculated using the Gompertz function and they represent the mean values of 6 mice for each case. When 10^6 fibroblasts were injected alone, no tumours were observed after 240 days.

Note that, when 10^5 cells were co-inoculated with 10^6 skin fibroblasts all mice developed tumours that appeared with a shorter delay time than was seen after 10^6 cells had been injected, but they grew at the same rate.

The lines are as follows:- 10^5 UCT-Mel 2 cells (o---o); 10^5 UCT-Mel 2 cells + 10^6 skin fibroblasts (■——■); 10^6 UCT-Mel 2 cells (▲——▲).

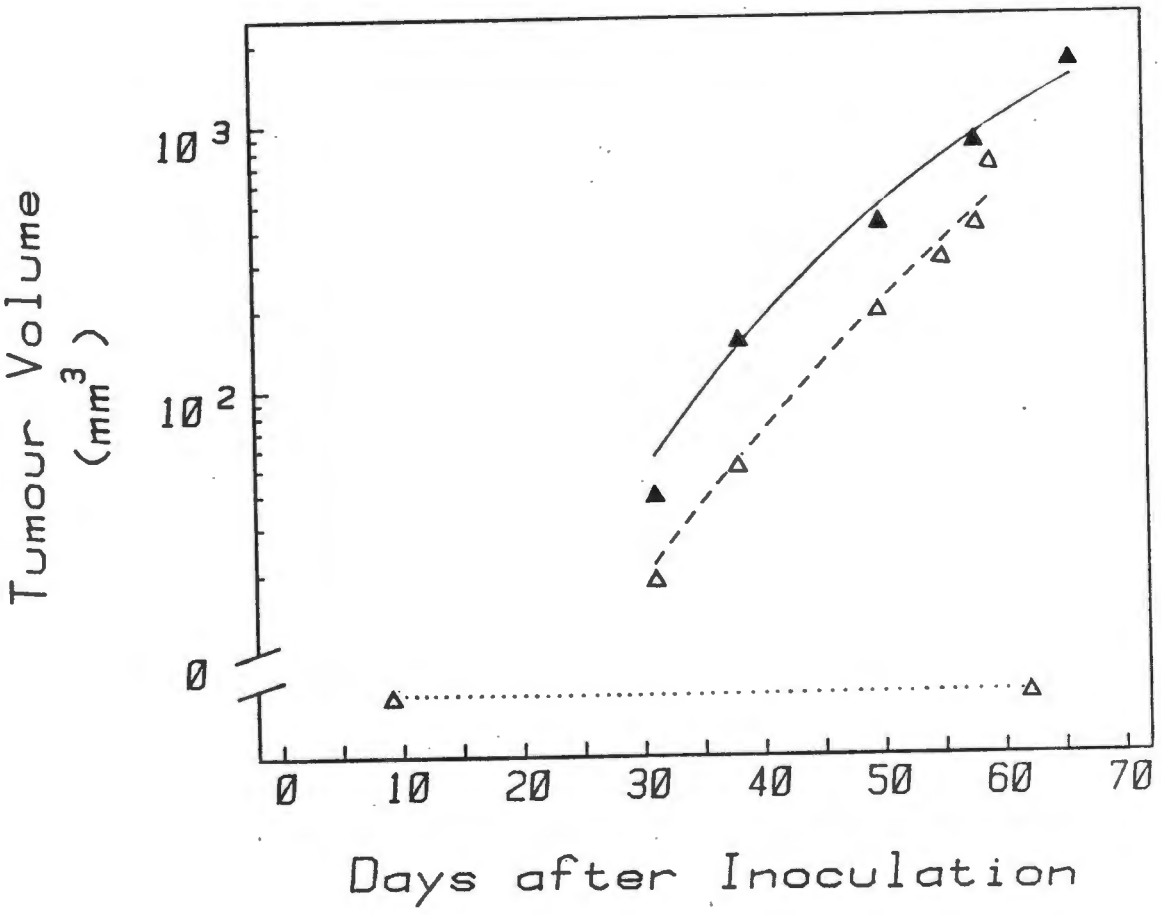


Figure 2.2

Tumour volume as a function of time for different inocula

Mice were inoculated subcutaneously with 10^5 or 10^6 UCT-Mel 3 melanoma cells, either alone or mixed with 10^6 viable skin fibroblasts. The points on the graph were calculated using the Gompertz function and they represent the mean values of 5 mice in the case of 10^6 melanoma cells injected alone or 3 mice in the case of 10^5 melanoma cells co-inoculated with 10^6 skin fibroblasts. When 10^6 fibroblasts were injected alone no tumours were observed after 195 days.

Note that, when 10^5 melanoma cells were co-inoculated with 10^6 skin fibroblasts 3 out of 5 mice developed tumours that appeared with a shorter delay time than was seen after 10^6 cells had been injected. However they grew at the same rate.

The lines are as follows:- 10^5 UCT-Mel 3 cells (△·····△); 10^5 UCT-Mel 3 cells + 10^6 skin fibroblasts (▲——▲); 10^6 UCT-Mel 3 cells (△--△).

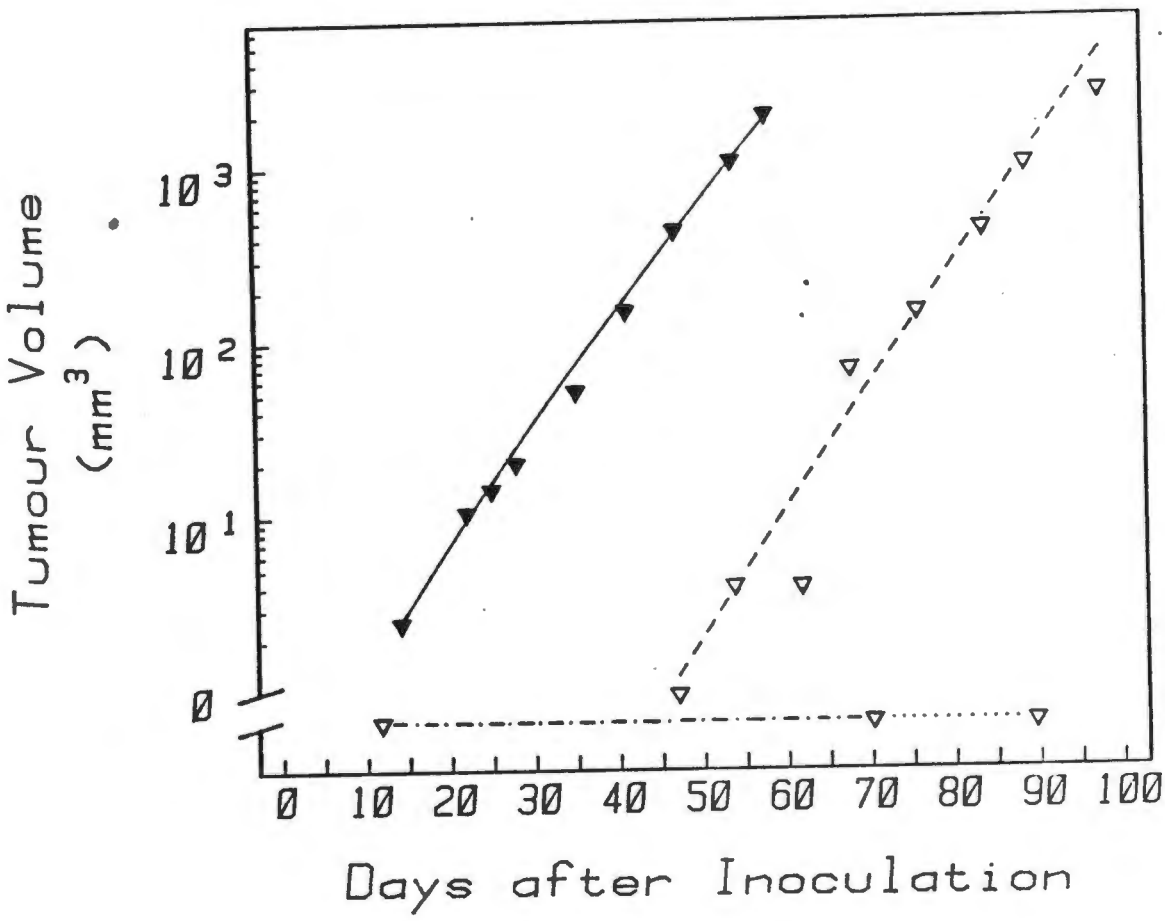


Figure 2.3

Tumour volume as a function of time for different inocula

Mice were inoculated subcutaneously with 10^5 UCT-Mel 2 melanoma cells, either alone or mixed with 10^3, 10^4 or 10^5 viable skin fibroblasts. The points on the graph were calculated using the Gompertz function and they represent the mean values of 2 mice in the case of 10^4 skin fibroblasts co-injected with melanoma cells or 4 mice in the case of 10^5 skin fibroblasts co-injected with melanoma cells.

Note that, 10^5 melanoma cells alone or mixed with 10^3 skin fibroblasts did not form tumours, whereas the same tumour cell inoculum with 10^4 or 10^5 skin fibroblasts produce tumours that grew at the same rate but with delay times inversely related to the number of complementing cells.

The lines are as follows:- 10^5 UCT-Mel 2 cells (▽·····▽); 10^5 UCT-Mel 2 cells + 10^3 fibroblasts (▽···▽); 10^5 UCT-Mel 2 cells + 10^4 fibroblasts (▽-----▽) 10^5 UCT-Mel 2 cells + 10^5 fibroblasts (▼——▼).

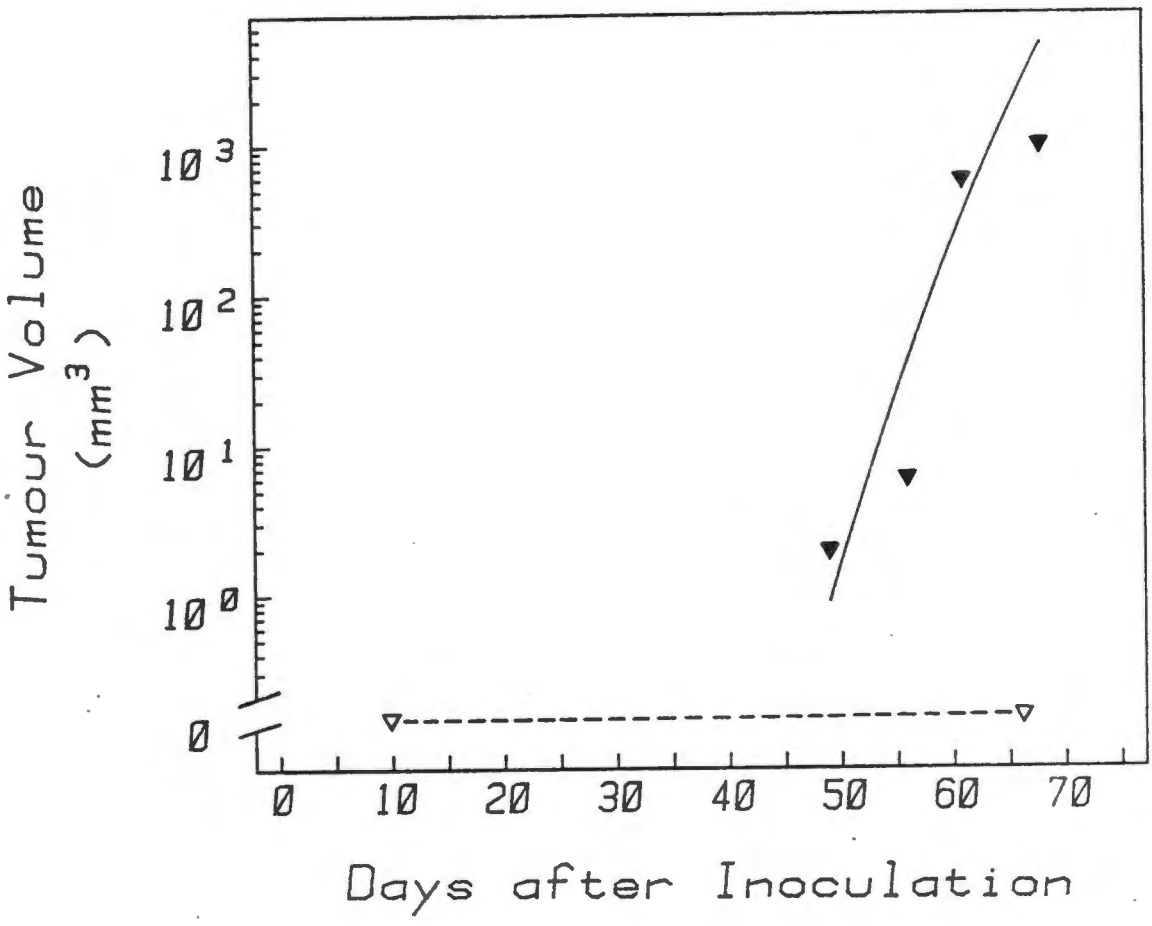


Figure 2.4

The effect of murine fibroblasts on the growth of melanomas in nude mice.

Mice were inoculated subcutaneously with 10^5 UCT-Mel 2 melanoma cells, either alone or mixed with 10^6 mouse embryo fibroblasts. The points on the graph were calculated using the Gompertz function and they represent the mean values of 4 mice.

Note that, 10^5 melanoma cells inoculated alone did not form tumours in mice; however when co-inoculated with 10^6 mouse embryo fibroblasts 4 out of 5 mice grew tumours with a long delay time after which they showed exponential growth.

The lines are as follows:- **10^5 UCT-Mel 2 cells (▽---▽); 10^5 UCT-Mel 2 cells + 10^6 mouse embryo fibroblasts (▼——▼).**

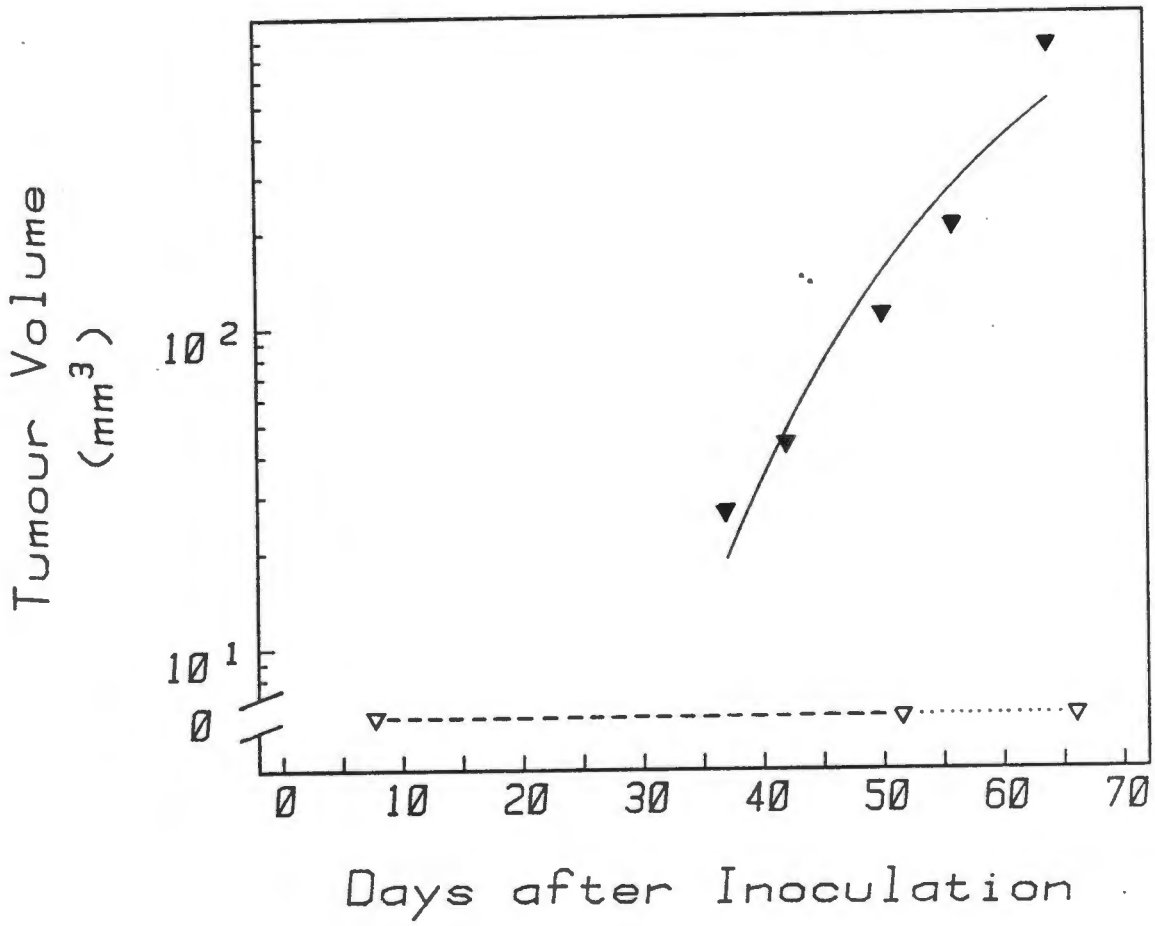


Figure 2.5

The effect of other cells on the growth of melanomas in nude mice.

Mice were inoculated subcutaneously with 10^5 melanoma cells, either alone or mixed with 3×10^6 UCT-Br 1 breast carcinoma cells. The points on the graph were calculated using the Gompertz function and they represent the mean values of 5 mice. When 3×10^6 breast carcinoma cells were injected alone no tumours were observed after 360 days.

Note that, when 10^5 melanoma cells were co-inoculated with 3×10^6 UCT-Br 1 breast carcinoma cells all mice developed tumours.

The lines are as follows:- 10^5 UCT-Mel 2 cells (▽·····▽); 10^5 UCT-Mel 2 cells + 3×10^6 UCT-Br1 cells (▼——▼); 3×10^6 UCT-Br1 cells. ▽----▽

blasts all of the mice developed tumours that appeared with a shorter delay time than was seen after 10^6 cells had been injected but they subsequently grew at the same rate. (Fig. 2.1). Exactly the same results were obtained with UCT-Mel 3, although in this case the differences in delay time were less striking (Fig. 2.2).

A dose-response effect was seen when different numbers of fibroblasts were used as complementing cells (Fig. 2.3). In this experiment 10^5 UCT-Mel 2 cells alone or with 10^3 fibroblasts did not cause tumours. The same tumour cell inoculum with 10^4 fibroblasts and with 10^5 fibroblasts produced tumours that grew at the same rate but with delay times that were inversely related to the number of complementing cells injected. Murine fibroblasts (Fig. 2.4) and breast carcinoma cells (Fig. 2.5) also functioned as complementing cells. Growth kinetics observed with the mouse fibroblasts were interesting in that a long delay time was observed before tumours appeared after which they grew more rapidly than UCT-Mel 2-derived tumours usually do. This experiment has not yet been repeated.

Inoculation at separate sites.

If, as seemed possible, the effects of the fibroblasts were mediated by some systemic mechanism involving the host, one might have expected that inoculation of the melanoma cells and complementing cells into different sites would produce enhancement of growth in much the same way when injected into the same site. This did not occur (Table 2.2); enhancement was only seen when the two cell types were inoculated as a mixture.

Sequential inoculation of potentially tumorigenic cells and complementing cells.

Révész, Littbrand and Modig (1967) were able to show enhancement of potentially tumorigenic cell growth even if complementing cells were injected into the same site as long as six weeks later and Wallace (1965) found that potentially tumorigenic cells from a C3HBA tumour implanted intramuscularly could be stimulated by complementing cells injected after 24 hrs. Hewitt,

Table 2.2

Inoculation of UCT-Mel 2 and adult skin fibroblasts as a mixture or into separate sites(a)

Group	Melanoma cells		Fibroblasts		Tumours
	Number	Site	Number	Site	No. of takes/ No. of mice
(b)					
I	10^5	sc r. flank	10^6	sc r. flank	5/5
II	10^5	sc r.flank	10^6	sc l. flank	0/2
III	10^5	sc r.flank	10^6	ip	0/2

(a) sc = subcutaneous

ip = intraperitoneally

(b) Mice in group I received melanoma cells and fibroblasts as a mixed inoculum.

Blake and Porter (1973) recorded an experiment in which murine potentially tumorigenic cells and complementing cells were implanted, in either sequence, into the peritoneal cavity. When these two injections were given concomitantly, tumours developed in 10/11 recipients. A high take rate was still seen if an interval of 3 hours separated the injections but the effect of the complementing cells was abolished if 23 hrs elapsed. When I gave sequential injections of melanoma cells and fibroblasts into the same site, I obtained the results shown in Table 2.3. Enhancement was only seen if a mixed inoculum was given.

Different cell types

In Table 2.4 I set out the effects seen when different cells were used as complementing cells. In each case 10^5 UCT-Mel 2 cells were inoculated with 10^6 complementing cells and the number of tumour takes was recorded. The non-tumorigenic breast carcinoma cell line, UCT-Br1 was used to see if cells other than fibroblasts would function as complementing cells. The skin fibroblasts used hitherto had been obtained from an adult male patient or from preputial tissue. Since (see Chapter 1) 10^5 UCT-Mel 2 cells were tumorigenic in male mice but not in females, it was necessary to see if female complementing cells would serve as well as male fibroblasts. For that reason fibroblasts isolated from a female foetal skin were used. One would have imagined that murine skin fibroblasts would have been abundantly present in subcutaneous tissue of the nude mouse and, had these been adequate to act as complementing cells, there would have been no need for extraneous complementing cells. This raised the possibility that murine fibroblasts, by virtue of a species difference, were functionally inadequate as complementing cells. Fibroblasts were thus prepared from nu/nu embryo tissue and these were used.

Finally, ethanol-fixed fibroblasts were used to see if metabolic viability was a pre-requisite of effective complementing cell function.

Table 2.3

Sequential subcutaneous injection (a) of 10^5 UCT-Mel 2 cells and 10^6 fibroblasts.

Interval	No. of takes/ No. of mice
2 min	0/2
18 hr	0/4
6 days	0/2
Mixed inoculum	5/5

(a) After the inocula of melanoma cells had been delivered, the needle was withdrawn completely and the fibroblasts were injected through a needle re-inserted, as closely as could be judged, through the same track and into the identical site.

Table 2.4

Efficacy of different cell types as complementing cells for 10^5 sub-tumorigenic inocula (10 cells) of UCT-Mel 2

Complementing cells	No. of tumours/ No. of mice
None	0/5
10^6 adult male fibroblasts	5/5
10^6 foreskin fibroblasts	5/5
$3x10^6$ UCT-Br 1	5/5
$3x10^6$ UCT-Br 1 alone	0/5
10^6 female foetal fibroblasts	5/5
10^6 ethanol-fixed fibroblasts (1)	0/5
10^6 mouse embryo fibroblasts	5/5

(1) Ethanol fixed fibroblasts were prepared as follows:

Adult male skin fibroblasts were detached from confluent monolayer cultures by gentle trypsinization and washed several times in RPMI medium. The cells were then pelleted gently (400g; 5 min; 20 °C) and resuspended in 1 ml of 70% ETOH. After 3 min of incubation at 37 °C the cells were pelleted once more, washed 3 times with PBS and resuspended in RPMI at the appropriate concentration for inoculation.

All of the cell types tested, with the notable exception of the ethanol-fixed cells, functioned as complementing cells.

Titration of the fibroblast effect

In a large experiment involving 98 mice, I performed an incomplete checkerboard titration of UCT-Mel 2 cells and fibroblasts to obtain the results summarized in Table 2.5.

The data show, once again, that 10^5 or less UCT-Mel 2 cells, when inoculated without fibroblasts, do not form tumours. Inocula of 10^6 tumour cells consistently did give rise to tumours. When 10^6 fibroblasts are used as complementing cells however, as few as 10 UCT-Mel 2 cells caused tumours in 3 out of 5 mice, and the enhancing effect of the fibroblasts is well seen in the other combinations. Of most interest is the observation that 10^4 melanoma cells, given with 10^4 fibroblasts gave rise to tumours in 4 out of 5 mice. Fibroblasts thus serve as more efficient complementing cells than do live tumour cells from the same line!

DISCUSSION

The data I present in this chapter demonstrate unequivocally that the successful transplantation of xenogeneic tumours into nude mice can be markedly enhanced by the addition of complementing cells to the inocula. The Révész phenomenon can thus be shown using human tumours in the nude mouse.

The characteristics of the Révész phenomenon that I have observed are, in all essential respects, similar to those observed by others who have studied experimental tumours inoculated into mice or rats. The complementing cells reduced the delay time without affecting the growth rate, indicating that the effect was to increase the number of cells in the inoculum that initiated tumour growth. Fibroblasts killed by ethanol fixation no longer served as complementing cells, indicating a need for metabolic viability.

Human fibroblasts isolated from adult skin, neonatal foreskin and female

Table 2.5

Titration of Révész effect with UCT-Mel 2 and fibroblasts

Fibroblasts in inoculum	Number of tumours/No. of mice inoculated UCT-Mel 2 cells in inoculum					
	10^1	10^2	10^3	10^4	10^5	10^6
0	0/5	0/5	0/5	0/5	0/5	5/5
10^3		0/5		0/5	0/5	
10^4		0/5		4/5	2/3	
10^5		1/5		3/5	4/5	
10^6	3/5	5/5	5/5	4/5	5/5	

(1) Ratios in each cell in the array show the number of mice that developd tumours as a fraction of the number of mice inoculated with each indicated combination of fibroblasts and UCT-Mel 2 cells.

foetal skin all functioned as complementing cells as did murine fibroblasts and a non tumorigenic line of breast cancer cells. This capacity would thus seem to be possessed by diverse cell types and unrelated to any readily identifiable attribute. Generally speaking, the biological research community tends to lose interest in phenomena that are non-specific or that cannot be associated with some readily identifiable and particular phenotypic characteristic. One report (Peters and Hewitt, 1974) has noted that lymphocytes and bone marrow cells failed to function as complementing cells but no attempt appears to have been made to follow up this observation and the Révész effect has not, in my opinion, received the attention it deserves. I believe that this is because research into the effect has had the wrong emphasis and that appropriate quantitative techniques for measuring the Révész effect have not been available. Given the supportive and immunologically propitious environment provided by the nude mouse host, it should, in theory, be possible to induce a tumour by the injection of a single malignant cell. In practice, however, this is usually not the case and one finds that the likelihood of a tumour developing is directly related to the number of cells injected. As a general rule, therefore, one may conclude that, for any particular set of experimental circumstances, a critical inoculum size range exists below which tumours will not occur and above which they will.

Although human tumours growing in the athymic mouse do not provide an entirely valid model for their behaviour in the natural host, the requirement for a critical inoculum size raised a number of questions of possible relevance to human tumour biology.

It is generally believed, for example, that human neoplasms are clonally derived from a single deviant mutant cell. What, then, are the factors that operate to sustain the proliferation of a tumorigenic cell under these spontaneous, natural circumstances yet do not operate to secure the survival of a presumably similar cell that has been injected subcutaneously into the athymic

mouse?

Are these "factors" generated locally by adjacent cells in the natural case and provided by complementing cells in the experimental situation? Is the success or failure of a metastatic "take" in any way related to the "complementing environment" in which a migrating cell is deposited? Is the Révész phenomenon, in short, a useful experimental model for studying the cellular modulation of a micro-environment that one may reasonably presume contributes to tumour progression or distant metastasis?

Use of the Révész effect with human neoplasms in the nude mouse thus offers considerable potential for studying a number of processes of interest to tumour biology. One may say with confidence, however, that this potential is unlikely to be realized until (a) it is generally appreciated that the interest lies in the complementing cell fraction itself and not entirely in the clonogenic potential of the tumour cell inoculum and (b) procedures are devised for quantitating complementing cell function. "Specificity" in biological systems has usually started out as a qualitative concept that becomes modified, by methodological progress, to become a quantitative one. Considered in this light our observation that adult skin fibroblasts and murine fibroblasts both function as complementing cells would indicate a lack of specificity. It is entirely possible however, that if the specific activity of these two cell types could be measured important differences would be discovered.

The results I present in Table 2.5 and Fig. 2.3 clearly demonstrate the feasibility of establishing such an assay and give some indication of the interesting possibilities that quantitative methods might offer.

It is evident, from the data in the table that, for each sub-tumorigenic dose of UCT-Mel 2 cells, a fibroblast dose-response effect could be discerned. When 10^5 UCT-Mel 2 cells were inoculated for example, no tumours developed if 10^3 or less fibroblasts were co-inoculated. Larger co-inocula of complemen-

ting cells resulted in an increasing proportion of takes until tumours formed in 100% of animals with 10^6 fibroblasts. When 10^4 or 10^5 potentially tumorigenic cells were injected, the TD50 for the complementing cells lay between 10^3 and 10^4 cells; when 10^2 potentially tumorigenic cells were injected the complementing cell TD50 rose to between 10^5 and 10^6 cells.

As it stands, the assay is probably not sensitive enough to detect subtle differences in complementing cell function. Useful statistical techniques are, however, available to improve the design and interpretation of bioassays of this sort (Finney, 1964), and it is my intention to exploit these in the near future.

The system was certainly sensitive enough to demonstrate the superiority of fibroblasts over melanoma cells as complementing cells.

A co-inoculum of only 10^4 fibroblasts complemented 10^4 UCT-Mel 2 cells to the extent that tumours developed in 4/5 animals receiving this combination. In remarkable contrast, none of the animals developed tumours when 9×10^4 viable UCT-Mel 2 cells were "co-inoculated" with the same number of potentially tumorigenic cells. One may conclude, therefore, that the fibroblasts were at least 9 times more effective than melanoma cells as complementing cells.

I have, as yet, no definitive explanation for the surprisingly superior performance of the fibroblasts in this experiment. Two possible reasons come immediately to mind, however, and it is my intention to explore them in the near future.

In the first place, it is well known that fibroblasts are able to synthesize somatomedins (Atkison et al., 1980; Clemmons et al., 1981) and other growth factors (Puck and Marcus, 1955) and it may simply be that, quantitatively, they released more of these compounds than did melanoma cells. If the Révész effect does, indeed, reflect a local "conditioning" of the tumour bed by complementing cells this would be an adequate explanation. Most growth

factors have molecular weights well below the limits for glomerular retention or slow diffusion, so that failure to demonstrate complementation by complementing cells inoculated at a distant site may simply reflect loss of these factors by excretion, dilution or degradation before they reached the potentially tumorigenic cell targets.

As alternative explanation for the superior fibroblast effect is suggested by two observations. The first was reported by Peter and Hewitt (1974) who showed that the Révész effect could readily be demonstrated when a thromboplastic extract of brain tissue or fibrin clots were substituted for complementing cells. They suggested, on the basis of these results, that the Révész effect was mediated by the thromboplastic action of complementing cells which caused local generation of fibrin. This enmeshed the tumour cells in a protective retaining web at the site of inoculation and that prevented their loss from the area and kept them in a local concentration that was high enough to ensure that their critical requirements for cooperative proximity were met. The second observation was reported by Hoal et al (1983) who showed that fibroblasts possess avid surface receptor-mediated mechanisms for the uptake and degradation of tissue plasminogen activator. This is a potent fibrinolytic enzyme that is known to be produced by UCT-Mel 2 (Wilson et al., 1980). It may well be, therefore, that the fibroblasts functioned well as complementing cells by virtue of their ability to inhibit fibrinolysis and so retard emigration of the potentially tumorigenic cells from the inoculation site. Since UCT-Mel 2 cells release tissue plasminogen activators, a much higher dose of the cells would be required to secure a tumorigenic balance between loss, by migration, from the site and the local persistance of a sufficient fraction of the inoculum to form an established tumour. This idea is readily amenable to experimental verification.

My observations of the Révész effect merit comment in one further and final respect: the growth of UCT-Mel 6 could not be enhanced by fibroblasts,

nor could that of UCT-Br1 . It is likely, from these results and from reading the literature on the subject that the Révész effect will only prove to be demonstrable with host-cell combinations that do allow some measure of tumour growth without complementing cells. This emphasizes the obvious need that exists for well-defined experimental systems. This need stems from the fact that the magnitude of the Révész effect seen in any given experimental situation represents the extent to which complementing cells mitigate the adverse circumstances under which an inoculum of potentially tumorigenic cells is implanted. The adversity may be a function of cell numbers (i.e. a small inoculum will not form a tumour), the environment (e.g. an irradiated tumour bed or a castrated host) or a combination of both (e.g. in the case of our UCT-Mel 2 cells that will not form tumours in female mice if 10^5 cells are injected whereas in males, inocula of this size are consistently tumorigenic).

Any quantitative study of the Révész effect, therefore, that requires measurement of a difference between implant behaviour with and without complementing cells, should attempt to maximize this difference by optimizing the condition of adversity. Clearly these should not be so severe (as in the case of UCT-Mel 6 or UCT-Br1) that clonogenic potential in the inoculum is effectively zero. Equally, this should not be so mild that complementing cell function is obscured by circumstances so propitious that complementing cells are not required.

I am unable to offer any useful explanation for the fact that UCT-Mel 6 and UCT- Br1 did not form tumours in the nude mice despite the fact that they clearly arose from malignant cells and they are capable of unrestrained permanent growth in vitro. UCT-Br1 cells were nevertheless able to function as complementing cells and the tumours that resulted when UCT-Mel 2 and UCT-Br1 were co-inoculated had the histological appearances of UCT-Mel 2 derived tumours.

CHAPTER 3

CHAPTER 3

THE EFFECT OF HORMONES ON THE GROWTH OF HUMAN MELANOMAS IN THE NUDE MOUSE.

Although a number of authors (Clayton, 1946; Pack and Scharnagel, 1951; Hadley, 1952; Byrd and McGanity, 1954; Stewart, 1955) had previously observed a deterioration in the clinical status of patients with malignant melanoma during pregnancy, it was (to judge from the number of times it has been sited) undoubtedly the paper published by Allen in 1955 that most effectively emphasized the possible adverse effects of pregnancy on the natural history of this disease.

He reported the remarkable case of a 38 year old woman who had a malignant melanoma excised from her right forearm. Approximately a year later she became pregnant and, as her pregnancy advanced, so the tumour disseminated widely. When seen in the third trimester, multiple blood-borne and lymphatic metastases were observed. One of these was removed and the diagnosis of an amelanotic melanomatous deposit was confirmed histologically. When seen two months after the delivery of her child, most of the nodules had disappeared. This improvement was sustained and she remained apparently free of disease for the next twelve years at least. This striking deterioration with pregnancy, and more significantly, complete remission following termination of the pregnancy, gave double proof of the association; it implied, quite clearly, that melanomas were subject to hormonal control in much the same way as were many other tumours; and it suggested an approach to the management of a tumour that was notoriously radio-resistant and unresponsive to other forms of therapy.

Pregnant women were known to be subject to cloasma gravidarum and pigmentary changes, and the findings in the serum and urine of pregnant women of substances that stimulate melanocytes supported the notion that humoral controls, possibly mediated by melanocyte stimulating hormone, might accelerate the course of the disease.

Further circumstantial support for the concept of endocrine control of the malignant melanoma phenotype came from observations that (i) prognosis in females is better than in males (White, 1959; Cochran, 1969; Nathanson et al., 1967b; Shaw et al., 1978; 1980); (ii) that the disease is rare before puberty (Sadoff et al., 1973); (iii) that approximately 50% of biopsy samples from melanoma patents showed oestrogen receptor activity (Fisher et al., 1976); (iv) that women who had used oral contraceptives had a higher incidence of melanoma than those who had not (Ellerbroek 1968; Beral et al., 1977; Lerner et al., 1979); and (v) that hormone therapy using drugs such as tamoxifen (Nesbit et al., 1979; Meyskens and Voakes 1980; Masiel et al., 1981), ablative procedures (Bodenham and Hale, 1972) or the administration of hormones (Levi and Lewison, 1952; Johnson et al., 1966; Sadoff et al., 1973; Fisher et al., 1976; Lederman et al., 1985) might alter the course of the disease for better or for worse.

Unfortunately, the effects of hormonal perturbation (e.g. pregnancy, menopause, contraceptive use or endocrine therapy) have been clinically and epidemiologically inconsistent (Shiu et al., 1976; Adam et al., 1981; Danforth et al., 1982; Helmrich et al., 1984; Stevens et al., 1980; Gill Grantley et al., 1984; Karakousis et al., 1980; Shaw et al., 1978; Neifeld and Lippman, 1980; Rampen, 1980, 1982) and the endocrine-dependent status of malignant melanoma is uncertain.

Factors that control cellular proliferation, differentiation and spread are important - not only in the therapeutic or prognostic context, but equally so as objects of scientific enquiry and it is somewhat surprising that so little work appears to have been done to assess humoral responsiveness of melanoma cells in the experimental systems that are available.

Numerous melanoma cell lines are available, for example, yet, as far as I am aware, only two reports have appeared in the literature to indicate that the <u>in vitro</u> effects of hormones on cultures of melanoma cells have been

studied.

Neifeld and Lippman (1980), in a review article on steroid hormone receptors and melanoma, state that, in their investigations of 21 melanoma cell lines, they were unable to detect estrogen binding activity. In 5 of the cell lines that they tested for estrogen responsiveness, none showed an effect of estrogenic or anti-estrogenic compounds on ^{3}H-thymidine or ^{14}C-leucine incorporation. No data were provided and this work has not subsequently appeared as a definitive report.

Chaudhuri et al., (1979) correlated hormone-binding activity with hormone responsiveness in 4 of 6 melanoma cell lines, but these observations could not be confirmed by others.

The lack of correlation between hormone-binding and *in vitro* response is not a matter of great concern since it has been clearly shown by Sirbasku (1979) that the biological effects of estrogen may be mediated, *in vivo*, by the indirect action of "estro-medins", or estrogen-induced "paracrine" growth factors that are derived from cells in the neighbourhood of the tumour cells rather than directly by effects upon the tumour cells themselves. Furthermore, the *in vitro* proliferation of most cells requires the presence of serum, and since sera contain variable concentrations of estrogens and other hormones, it is difficult to achieve experimental conditions in which cellular behaviour in the presence of a single hormone can be reliably compared with that in the absence of the compound.

In vitro studies are thus beset with problems in interpretation and experimental execution that have probably made them unattractive to research workers.

The nude mouse system circumvents many of these problems. As I have indicated in Chapter 1, xenografts of human melanoma cells usually grow well in the athymic mouse to produce tumours that are remarkable for their similarity to the parent tumour from which they were derived. Sustaining

host-cell functions (and presumably these include estro-medin synthesis) are available to support tumour development in an environment that is more physiological than that of the petri-dish; and it is likely that these functions would be susceptible to endocrine control. Finally, it is relatively easy to manipulate the endocrine status of the mouse by ablation or supplementation in ways that have potential clinical relevance.

Exploitation of these useful features of the xenograft model to study hormone responsiveness of breast carcinoma and choriocarcinoma are exemplified by the report of Brünner and Visfeldt (1982), Shafie and Grantham,(1981) and Kameya et al.,(1976) respectively.

Few authors, however, seem to have availed themselves of the advantages that the nude mouse model has to offer for the study of hormone-dependence of human malignant melanoma. Beattie et al.,(1979) studied two cell lines, one of which bound estrogen and the other not. They found that estrogen receptor activity in the line correlated with an inhibitory effect of estrogen on the growth in the nude mouse in as much as the receptor positive line grew more slowly in females with the estrogen than the estrogen receptor negative line whereas these differences were not seen in males. Furthermore, alternate-day subcutaneous estradiol administration retarded growth of the estrogen receptor positive lines in males. Giovanella et al.,(1974) published an abstract in 1974 in which they stated that a melanoma line derived from a male patient grew preferentially in female mice and Kozlowski et al.,(1984) demonstrated an increase in the metastatic potential of two human melanoma cell lines as a result of estradiol administration.

I felt that more could be done to resolve the question of human responsiveness of melanomas using the nude mouse system and in this chapter I record my experimental observations with the xenograft model.

MATERIALS AND METHODS

Mice

Athymic nude mice that were homozygous for the nu and the xid genes on the NIH Swiss background (Azar et al., 1980) were raised and maintained as described in detail in Chapter 1.

When the experiment called for castrated animals, the ovaries and the testes were removed under sterile conditions from 5-6 week old animals that were anaesthetized with ether and held in the supine position.

Each ovary was delivered, attached to its mesosalpinx and fallopian tube, through a lower abdominal midline incision. The gonad was excised after ligation, with a purse string suture, of the ovarian vessels and attachments to the mesosalpinx and the associated structures were returned to the peritoneal cavity. The skin incision, abdominal wall and peritoneal cavity were then closed with clips.

In the case of males, the testes were delivered through a ventral incision in the scrotal skin and removed by severing the spermatic cord and vessels distal to a purse-string ligature. The scrotal incision was then closed with a clip.

Sham-operated animals were anaesthetized and had the skin incisions without castration. The skin clips were removed from all animals after 4-5 days.

Despite the immunodeficient status of the animals they withstood the procedures without complications and infection was not a problem.

Experiments were started when the animals were 8-10 weeks old.

Hormonal therapy

Hormones were administered to the animals as pellets that were implanted subcutaneously and that gave sustained, slow release of the hormone over a prolonged period of time. The pellets were obtained from Innovative Research

of America, Gaithersburg, Maryland 20877, USA and came with the specifications summarized in Table 3.1.

Table 3.1

Specifications of pellets supplied by Innovative Research of America.

Cat. No.	Active ingredient	Effective-dose delivery time(1)
E121	17β-Estradiol	21 days
A161	5 -Dihydrotestosterone	21 days
P131	Progesterone	21 days
E351	Tamoxifen	21 days

(1) Determined by measurement of serum hormone concentration as a function of time after implantation into castrated animals.

The pellets, which came supplied as discs with a diameter of approximately 2 mm and a thickness of 1 mm were implanted under the midline skin of the back in the scapular region or, in some cases, near the base of the tail. To do this, I made a small (approximately 2mm) nick in the skin of the anaesthetized animal with pointed scissors. Through this I introduced a trochar and cannula and advanced it, in the subcutaneous plane, until the tip was approximately 2.5 cm from the incision and at the point where I wished the pellet to reside. The trochar was then withdrawn and the pellet was deposited in the tissues at the end of the cannula, by inserting it into the cannula, replacing the trochar and gently advancing the trochar until it was fully home. The assembled trochar and cannula were then removed from the animal and inspected to ensure that the pellet had been delivered intact. The small skin incision was closed by apposition. It did not require clipping or suturing. Control animals received pellets that contained the compressed vehicle and all other ingredients save the active hormone.

The implantation of sustained-release pellets proved to be a remarkably effective, reproducible and convenient way of administering long-term hormonal treatment.

Cells

The melanoma cells used for the experiments and their preparation for inoculation have been described in detail in Chapter 1 and Appendix section (A1 to A6).

Experimental protocols

For each experiment to be described in this Chapter I used a group of mice, all of whom had been born within the same two-week period. Each procedure (e.g castration, implantation of pellets, inoculation of tumour cells or measurement of tumour size) was performed on all members of the experimental group on the same day. Within any experiment, therefore, circumstances were consistent in as much as they related to the batch of animals, pellets or inoculated cells that were used or to the environmental conditions that prevailed during the course of the experiment.

I divided each experimental group into control and test sub-groups according to a factorial protocol that enabled me to test the effect of up to three variables in the same experiment, so economizing the use of valuable animals. This protocol is illustrated by the experiment in wich I wished to examine the effect of sex (i.e. male or female), castration and dihydrotesterone (DHT) therapy alone or in all possible combinations. Since, in this case, there were three variables (sex, castration and DHT therapy) each with two alternatives (respectively male/female; sham operated/castrated; control pellet/DHT pellet) this gave $2^3 = 8$ combinations. I allocated 5 mice to each combination, thus the experiment comprised 40 animals. In other experiments (as described in the Results section) as few as 3 or as many as 6 mice were used in each combinatorial subgroup.

The factorial experimental protocol gives results that are ideally suited to statistical evaluation by the analysis of variance and it was for this reason that I designed most of my experiments in this way. As I point out in the Results section, however, parametric statistical techniques based on the analysis of variance did not accommodate cases where tumours failed to develop and, in retrospect, a different experimental design may have been preferable.

In most cases pellets were implanted when the animals were 8 - 10 weeks of age and melanoma cells were inoculated the following day. The animals were marked and examined weekly for at least six months. When tumours developed, their volumes were recorded weekly. Gompertzian growth curves were fitted, by repeated least-squares regression, to these data and from these doubling times required to reach a volume of 200mm^3 (Td 200) and latency times (i.e. the time taken to reach a size of 100mm^3 - T(del)100) were calculated. These methods are described in detail in Chapter 1.

Estrogen and progesterone receptor measurements

Estrogen and progesterone binding activity in the melanoma cells used in these experiments were assayed by the method of Zava et al. (1976) using ^{3}H-estradiol and ^{3}H-R5020 (a specific progestin).

RESULTS

Hormonal control of the growth of melanoma xenografts

All told, 10 experiments were done to determine whether or not the growth of human melanoma cell xenografts was in any way influenced by the endocrine status of the recipient mice. These experiments involved the inoculation of different numbers of melanoma cells from different cell lines into a total of 431 mice that had been prepared to receive the inocula by choice of sex (M/F), castration or sham operation (Cx/Sx) or the implantation of a pellet of 17B estradiol (E2), dihydrotestosterone (DHT), progesterone (PROG) tamoxifen (TAM),or a vehicle control.

As described in the methods section, these preceding variables and their corresponding reference controls were assigned either singly or, where possible, in factorial combination. Most experiments comprised 8 to 12 sub-groups, each of which usually contained 5 mice and observations were recorded weekly on each mouse for periods that varied from several weeks (in the cases where tumours developed and grew) to six months (in those cases where they did not).

I have thus accumulated several thousand observations and the question arises as to how to present a concise and coherent summary of these results that will best convey their substance and import.

My initial experiments led me to believe that growth curves constructed from in vivo measurement could be described with Gompertzian parameters with sufficient accuracy for me to use these paramaters (or the delay times and doubling times derived from them) to measure the effects of hormonal manipulations and for me to apply standard parametric statistical procedures to the estimation of the significance or differences that I observed. Unfortunately my expectations in this regard were not fulfilled.

Deviations from precise Gompertzian kinetics were quite frequent and, when these occurred, it was often obvious, from inspection, that the fitted

curves did not portray the data accurately. Furthermore, it frequently happened that, in one or more mice in a group, tumours did not develop. In these cases delay times and the doubling times were infinite and the analysis of variance did not take kindly to infinite values.

Procedures may well exist to handle results that combine rational and nominal values in the same data set, but the statistical advice to which I had access did not suggest a ready solution. I have, therefore, chosen to present the data in the form of individual growth curves without statistical analysis. For each experiment I have included a short table that summarises the experimental design and tumorigenicity data and the average t(del)100 and Td200 for tumours within a particular group where it was possible to compute these. In certain cases the effects of hormonal manipulation were most clearly seen when groups were compared at a particular time after inoculation. To illustrate these time selected data I have constructed bar graphs depicting averages for each group. These graphs emphasize differences on a linear scale that are not always apparent when tumour sizes are presented for visual comparison on a logarithmic scale in the growth curve plots. Only those data that are convincing and that are pertinent to the conclusions that I wish to draw are presented here. A full account of the experimental results is presented in the Appendix Table A9-A12.

The effects of hormonal manipulation were most clearly documented in the following series of experiments.

Experiment (i)

Table 3.2

Sex	Males		Females	
Operation	Sx	Cx	Sx	Cx
Treatment	-	-	-	-
Tumour No.	4/4	0/4	2/5	2/5
T(del)100	97	-	66	48
Td 200	8	-	7	4

Male (8) and female (10) mice were each divided into two groups (Table 3.2). Mice in one group had the gonads removed while the corresponding control groups were submitted to the sham-operation. UCT-Mel 2 cells (10^5) were inoculated into the 4 groups three weeks later and the animals were observed for tumour development. Tumours developed in all of the sham-operated male mice but in none of the castrated males (Fig. 1a). Only 2/5 female mice in the sham-operated group developed tumours; the oophorectomised animals showed the same incidence of tumours. Tumours formed in female mice did so earlier in castrated animals than in sham-operated females (Fig. 1b). It is also evident from Figs. 1a and b that tumours developed in females approximately one month earlier than they did in males. Once established, however, the tumours grew at the same rate in males and females. Fig. 1c depicts the size of the tumours in castrated and control male and female mice 100 and 66 days after inoculation respectively. The tumours which grew in the ovariectomized females were far larger ($\pm$ 680mm^3) than the tumours in the sham-operated female mice ($\pm$ 30mm^3) when measured 66 days after inoculation of tumour cells.

It thus appeared, from this experiment, that an inoculum of 10^5 UCT-Mel 2 cells was marginally tumorigenic in females, uniformly tumorigenic in normal

FIGURES 1a and 1b

Figure 1a

Growth of human melanomas in castrated and sham-operated male nude mice.

Male mice were inoculated subcutaneously with 10^5 UCT-Mel 2 cells (124') on day 0 and the tumour volumes determined at weekly time intervals. The shaded area represents the range of tumour volumes of 4 control mice. The lines terminating in encircled numbers at the bottom of the figure indicate mice (4) in which tumours failed to develop.

(·····) castration (4)

Note that, tumours failed to grow in castrated male mice.

Figure 1b

Growth of human melanomas in ovariectomised and sham-operated female nude mice

Female mice were inoculated subcutaneously with 10^5 UCT-Mel 2 cells (124') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volume as a function of time for 2 individual ovariectomised mice. The shaded area represents the range of tumour volumes for 2 control mice. The lines terminating in encircled numbers at the bottom of the figure indicate animals in which tumours failed to develop.

(·····) castration (3)

(————) control (3)

Note that castration reduced the delay time of the tumour; however the tumorigenicity was similar in both cases.

Figure 1a

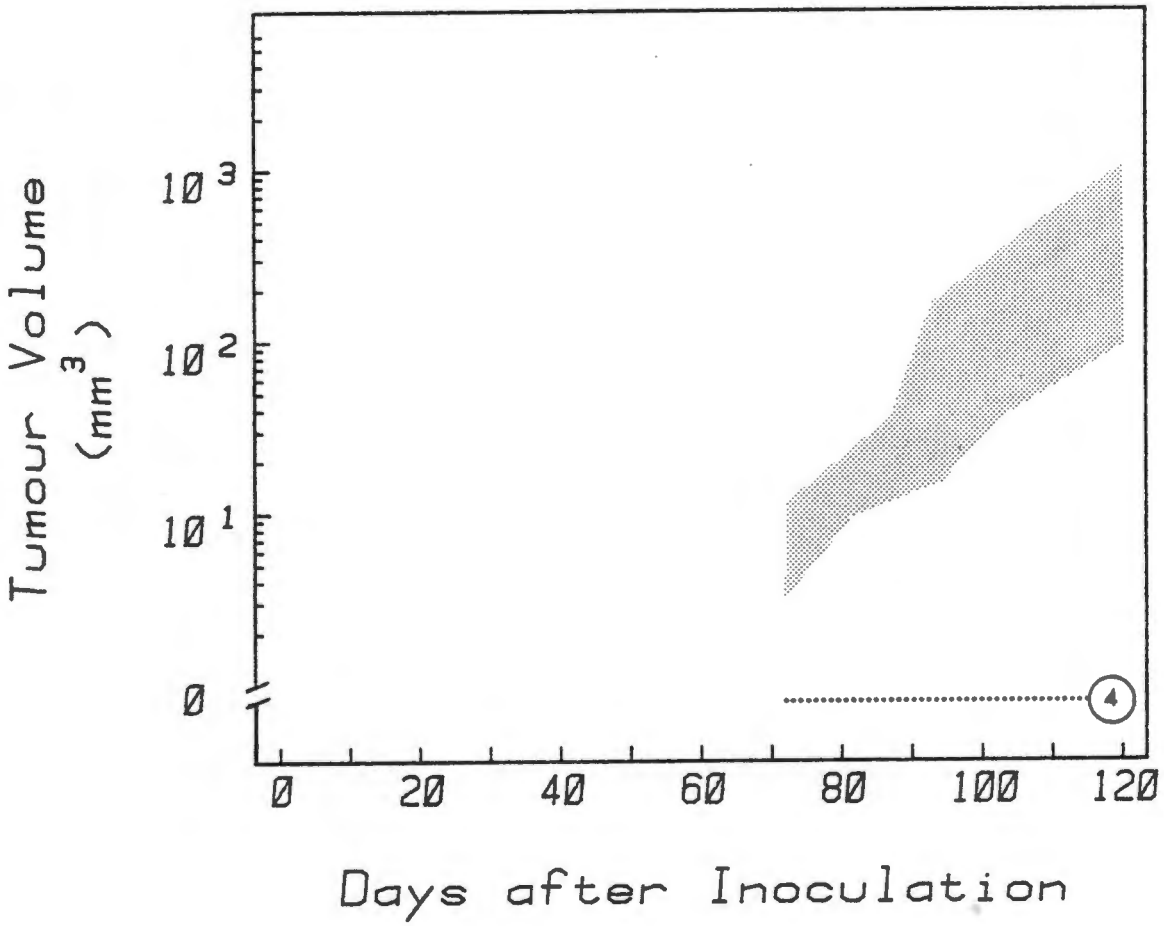
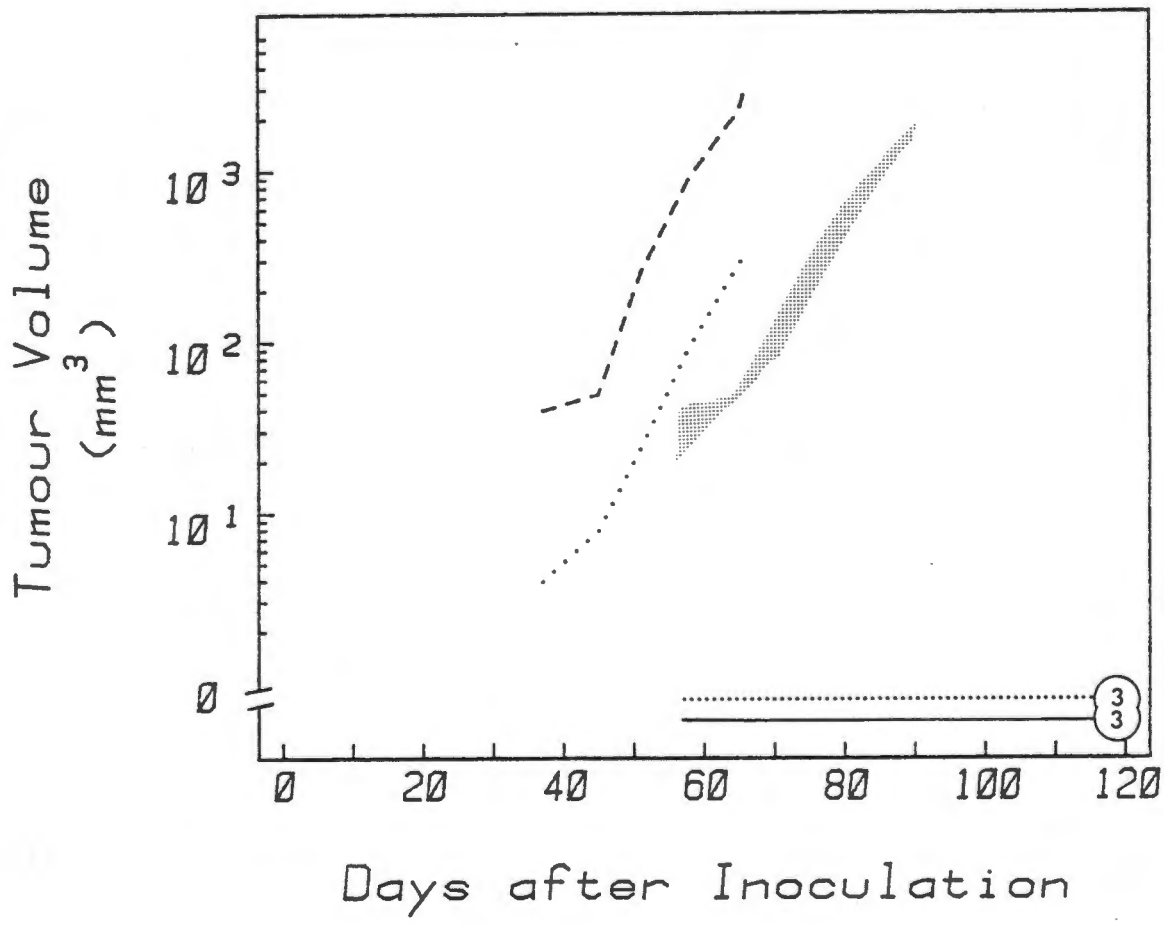


Figure 1b



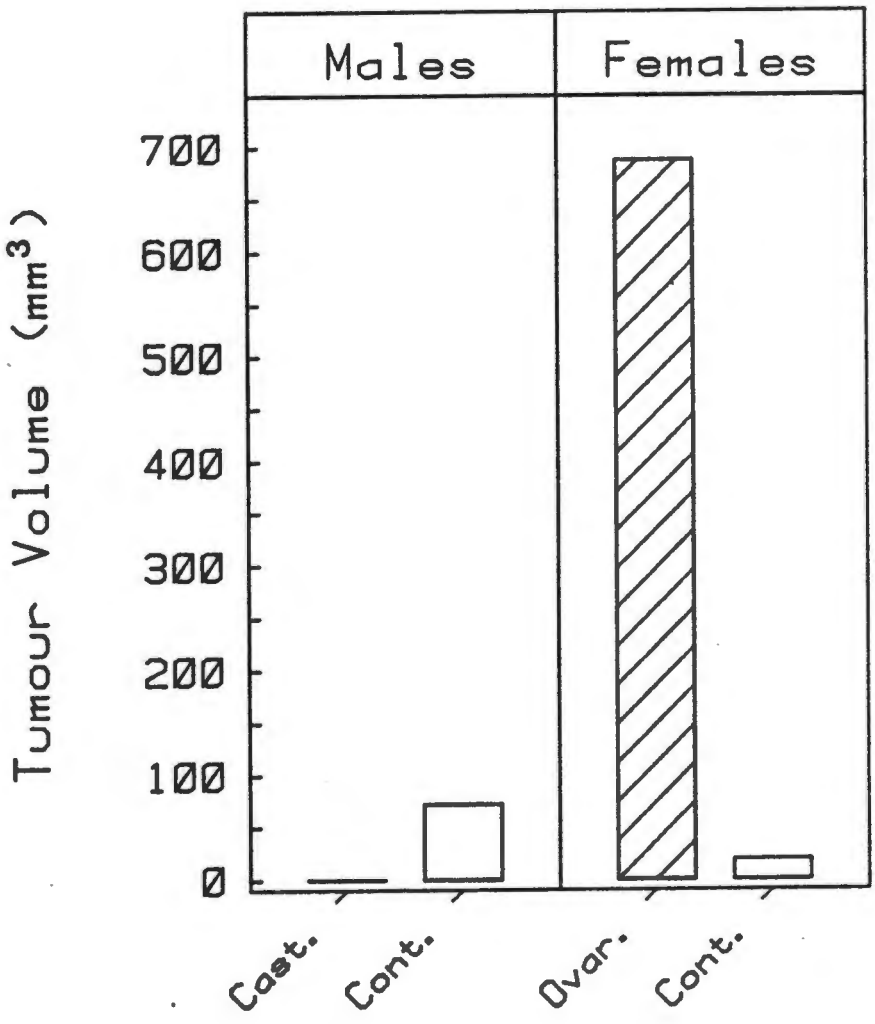


Figure 1c

The effect of castration on the growth of UCT-Mel 2 tumours in nude mice

Male and female mice were castrated and 3-4 weeks later 10^5 UCT-Mel 2 melanonoma cells (124') were inoculated subcutaneously. Sham operated mice were similarly inoculated. Tumour volume was measured 66 days later in the case of female mice or 100 days later in the case of male mice.

Note that castration inhibited the growth of tumours in male mice whereas in females the growth of the tumours was stimulated by this procedure.

▨ - castrated

□ - sham-operated

males and non-tumorigenic in castrated males and would thus provide a useful basis for developing a semi-quantitative assay of host environmental factors required for melanoma xenograft growth.

It should be noted that, at the time at which the experiments reported in chapter 2 were performed, inocula of 10^5 UCT-Mel 2 cells were consistently non-tumorigenic. In these experiments they were now marginally so. I have no good reason to offer for this subtle variation in tumour incidence; the reason may lie with the cells or with the animals.

I, therefore, performed the following experiments with this intention with the earlier experiments in mind.

Experiment (ii)

Table 3.3

Sex	Males		
Operation	Sx	Cx	
Treatment	Cnt	Cnt	DHT
Tumour No.	2/5	0/5	2/5
T(del)100	59		77
Td 200	14		Not done

Fifteen male mice were divided into three groups of five each (Table 3.3). The first group was sham-operated; mice in the second group were castrated; and those in the third group were castrated and treated with DHT. Each mouse received an inoculum of 1.5×10^5 UCT-Mel 2 cells.

As observed previously none of the castrated males developed tumours. On this occasion, however, tumours developed in only 2/5 sham-operated males. Androgen treatment of castrated males increased the take rate from 0/5 to 2/5.

Experiment (iii)

Table 3.4

Sex	Males				Females			
Operation	Sx		Cx		Sx		Cx	
Treatment	Cnt	DHT	Cnt	DHT	Cnt	DHT	Cnt	DHT
Tumour No.	1/5	2/5	3/5	3/5	1/4	2/5	1/5	5/5
T(del)100	40	58	59	44	50	54	41	57
Td 200	12	15	5	Not done	7	11	5	9

In this experiment, summarized in Table 3.4, I studied the effects of sex, castration and DHT supplementation in all combinations on the ability of $2x10^5$ UCT-Mel 2 cells to cause tumours. Here oophorectomy plus DHT supplementation provided the most favourable (5/5) host environment for tumour development. Surprisingly, in this experiment, sham-operated males showed a lower take rate (1/5) than did castrated males (3/5).

DHT did not increase tumorigenicity in orchidectomised males.

Experiment (iv)

Table 3.5

Sex	Males						Females					
Operation	Sx			Cx			Sx			Cx		
Treatment	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT
Tumour No.	3/5	5/5	3/4	4/5	5/5	4/4	1/5	4/5	3/5	3/4	4/4	4/5
T(del)100	68	28	43	32	27	31	37	30	38	39	26	36
Td 200	7	5	10	5	6	13	6	5	6	7	10	7

For this experiment I inoculated $4x10^5$ UCT-Mel 2 cells into each of 56 mice allocated to the twelve treatment groups identified in Table 3.5.

The effects of estrogen implantation were striking. Tumours grew in only 1/5 females (Fig. 2a) and 3/5 males (Fig. 2b) whereas 4/5 females (Fig. 2a) and 5/5 males (Fig. 2b) developed tumours after E2 implantation.

An effect of E2 was also seen in castrated animals. Oophorectomised females supported tumour growth in 3/4 cases but in 4/4 cases after E2 implantation (Fig. 2c). In males, E2 pellets increased the take rate from 4/5 to 5/5. As can be seen from Fig. 2c and 2d and Table 3.5, the latent period for tumour development was shorter in E2-treated animals. Castrated females required on average, 39 days for a tumour of 100mm^3 to be reached whereas E2 supplementation reduced this period to 26 days. Castrated males treated with estrogen required 27 days for a similar sized tumour to develop compared to 32 days for castrated animals and 68 days for intact male mice.

Fig. 2e shows the size of tumours in the various groups of mice 36 days after inoculation of cells. The stimulatory effect of estrogen on tumour volume can be noted in all groups of mice.

DHT treatment increased the tumour take rate in all groups. Tumour volume was also increased by DHT although not as profoundly as with estrogen (Fig. 2e).

FIGURES 2a and 2b

Figure 2a

Growth of human melanomas in estradiol treated female nude mice

Female mice were inoculated subcutaneously with 4×10^5 UCT-Mel 2 cells (124') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 4 individual mice treated with estradiol. One of the mice treated with estradiol did not develop a tumour. The continuous line represents tumour volumes for 1 out of 5 control mice. The lines terminating in encircled numbers at the bottom of the figure indicate mice in which tumours failed to develop.

(--------) estradiol treated (1) (————) control (4)

Note that estradiol enhanced the growth of the tumours in female mice.

Figure 2b

Growth of human melanomas in estradiol treated male nude mice

Male mice were inoculated subcutaneously with 4×10^5 UCT-Mel 2 cells (124') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volume as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 3 control mice. The line terminating in an encircled number at the bottom of the figure indicates control mice (2) in which tumours failed to develop.

(————) control (2)

Note that estradiol reduced markedly the delay time of the tumours and stimulated tumour growth.

Figure 2a

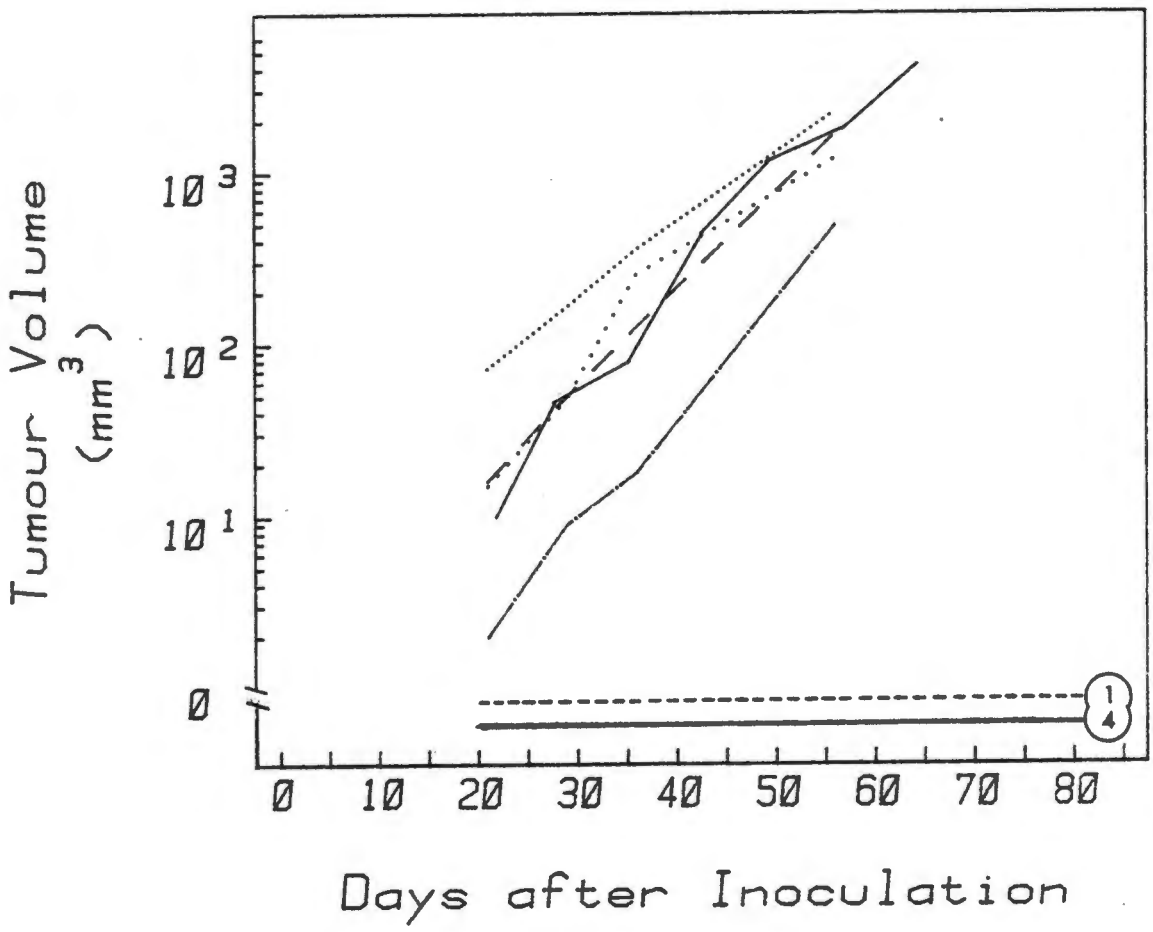


Figure 2b

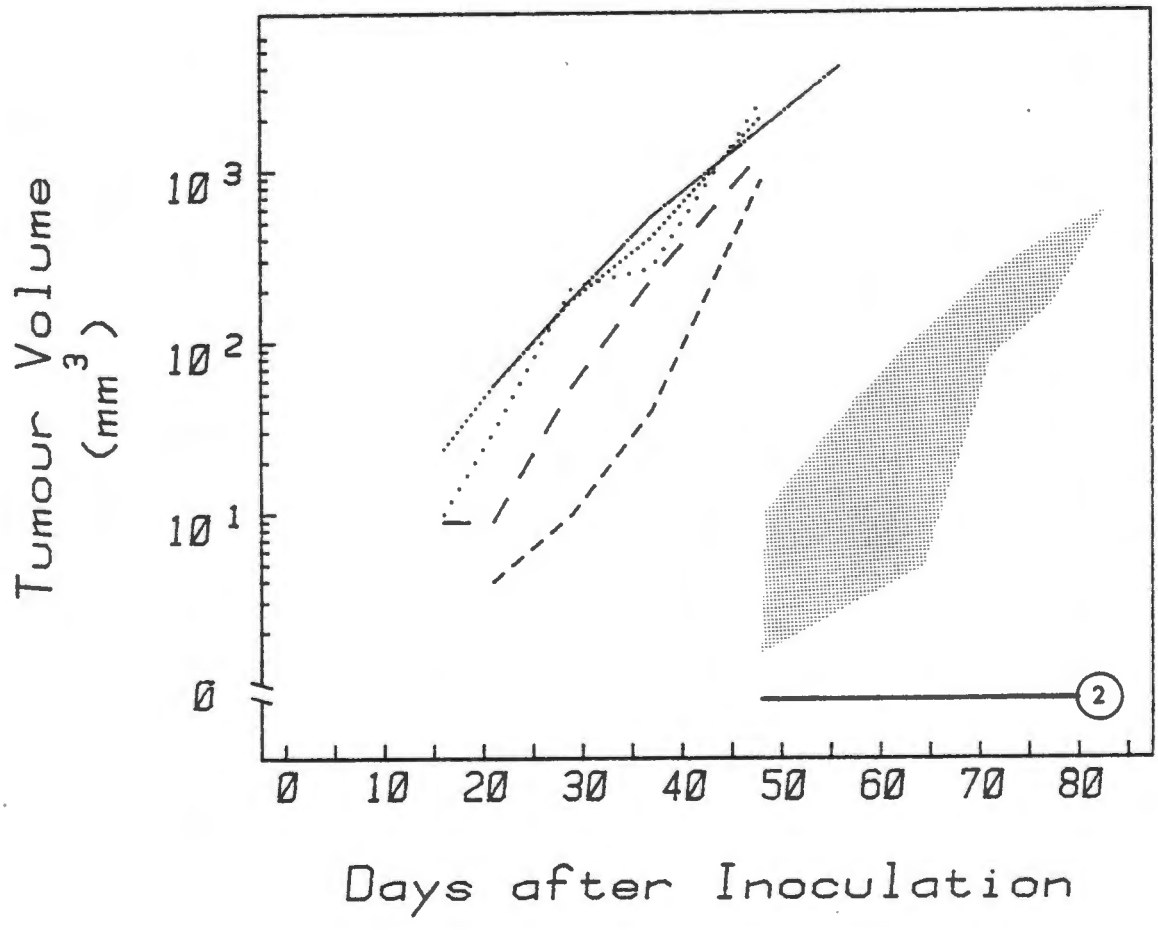


FIGURE 2c and 2d

Figure 2c

Growth of human melanomas in ovariectomised and estradiol treated female nude mice

Female mice were inoculated subcutaneously with 4×10^5 UCT-Mel 2 (124') cells on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volume as a function of time for 4 individual ovariectomised mice treated with estradiol. The shaded area represents the range of tumour volumes for 3 ovariectomised control mice. The line terminating in an encircled number at the bottom of the figure indicates the control mouse (1) in which tumours failed to develop.

(————) control (1)

Note that estradiol, as shown previously, increased the growth of the tumours.

Figure 2d

Growth of human melanomas in castrated and estradiol treated male nude mice

Male mice were inoculated subcutaneously with 4×10^5 UCT-Mel 2 cells (124') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting estimated tumour volumes as a function of time for 5 castrated mice treated with estradiol. The shaded area represents the range of tumour volumes for 4 castrated control mice. The line terminating in an encircled number at the bottom of the figure indicates the control mouse (1) in which tumours failed to develop.

(————) control (1).

Note that estradiol enhanced the growth of the tumours and reduced the delay time; however tumours in both groups developed at the same rate.

Figure 2c

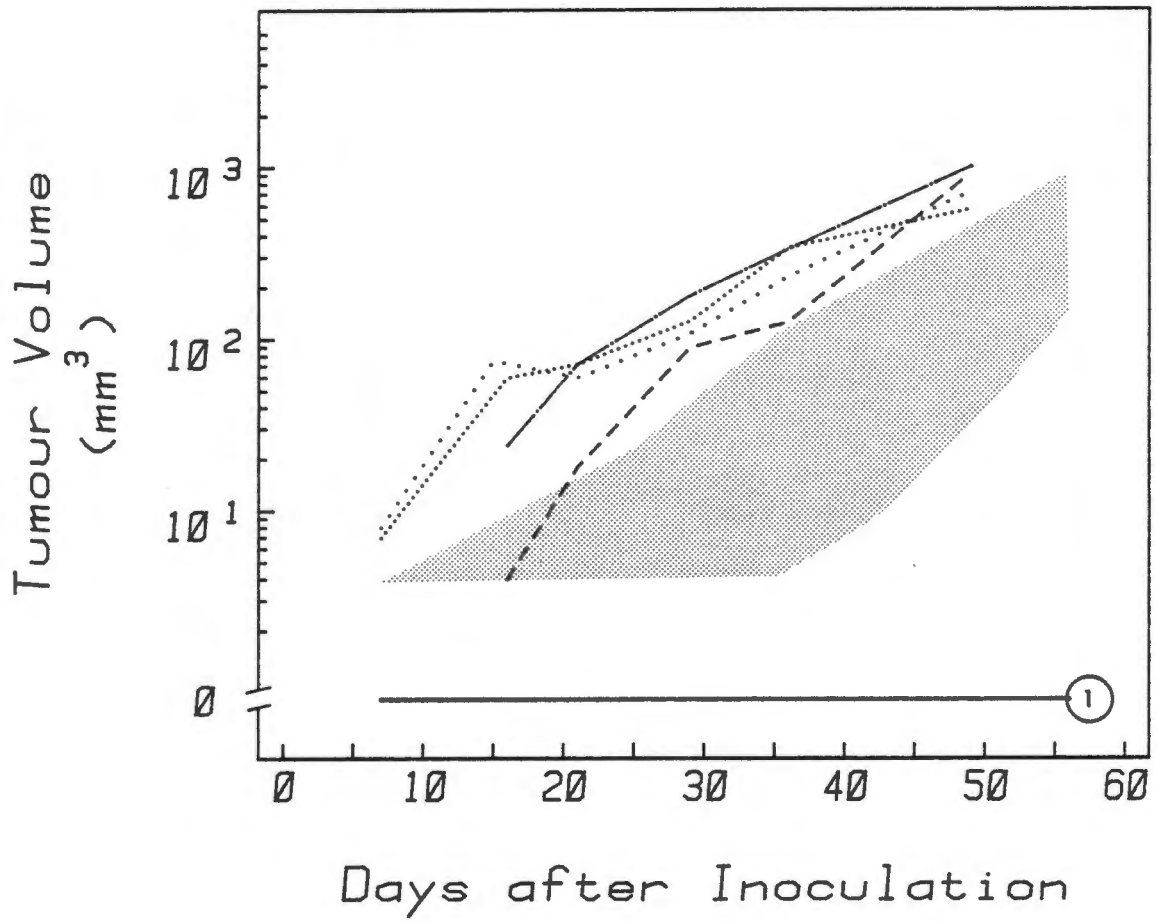


Figure 2d

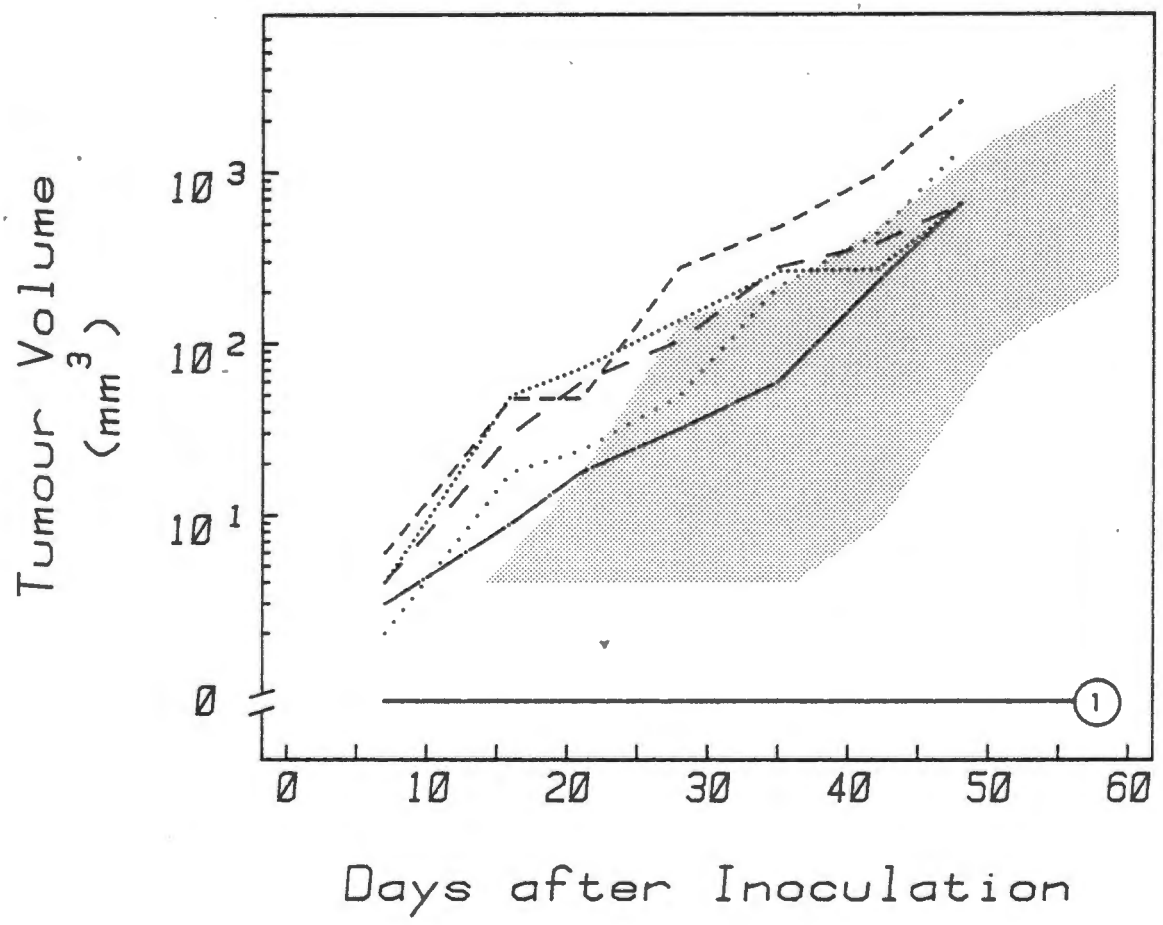


FIGURE 2e

Figure 2e

The effects of castration and hormonal therapy on the growth of UCT-Mel 2 tumours in nude mice.

Male and female mice were castrated and 3-4 weeks later dihydrotestosterone (DHT) or estradiol (E2) pellets were implanted subcutaneously. Twenty-four hours later 4×10^5 UCT-Mel 2 cells (124') were inoculated into castrated and sham-operated and control animals. Tumour volumes were measured 36 days later.

Note the following:-

(a) In all 4 groups of mice hormonal therapy had an effect on the growth of UCT-Mel 2.

(b) Both DHT and estradiol stimulated growth of tumours in untreated male mice.

(c) Castration alone resulted in increased tumour size in male and female mice. This was further enhanced in both sexes by estradiol implants.

▨ - castrated ☐ - sham-operated.

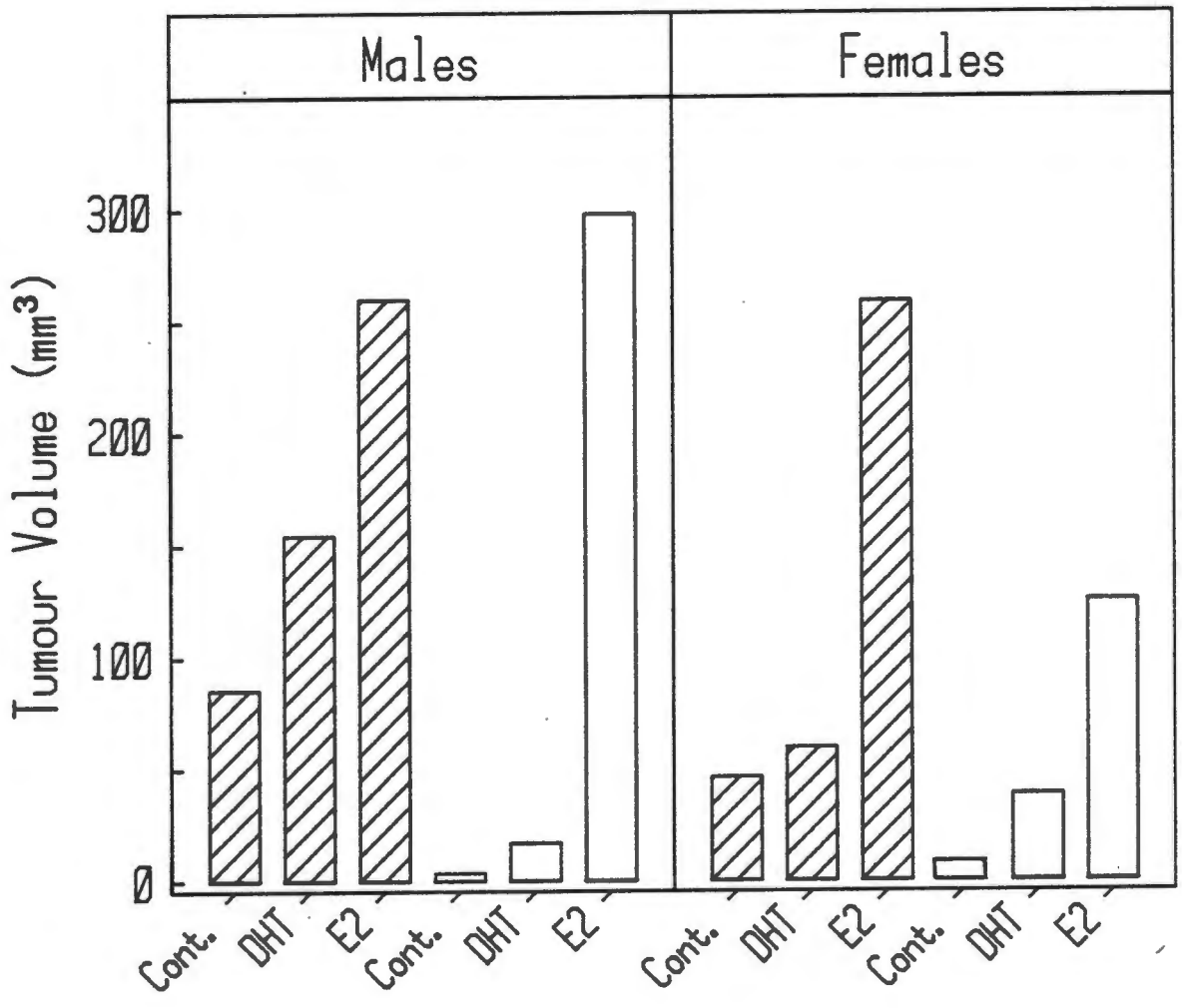

Males
Females
Tumour Volume (mm³)
300
200
100
0
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2

Figure 2e

Experiment (v)

Table 3.6

Sex	Males						Females					
Operation	Sx			Cx			Sx			Cx		
Treatment	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT
Tumour No.	5/5	5/5	5/5	3/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5
T(del)100	38	33	36	41	31	14	40	34	29	43	25	30
Td 200	7	4	10	3	6	6	5	6	7	5	5	7

This experiment (Table 3.6) was identical to Experiment (iv) save for the inoculation of 10^6 UCT-Mel 3 cells in place of $4x10^5$ UCT-Mel 2 cells.

In this experiment E2 had a slight effect on tumour growth in sham-operated female or male animals (Figs. 3a and 3b) whereas it had a pronounced effect in castrated animals (Fig. 3c and 3d). Melanoma cells inoculated into oophorectomised females treated with E2 formed tumours with a shorter delay time (25 days) than did cells injected into untreated oophorectomised females (43 days). E2 had no effect on tumorigenicity in this group as tumours grew in all inoculated animals (Fig. 3c, Table 3.6).

E2 had a pronounced effect on melanoma growth when administered to orchidectomised males and ovariectomised females (Fig. 3c and 3d). The take rate in orchidectomised males was increased from 3/5 to 5/5 by the hormone and the T(del)100 was 31 days as opposed to 41 days in the absence of E2. As can be noted from Fig. 3d tumours also developed much earlier in the presence of this hormone.

Fig. 3e illustrates the effect of estradiol and DHT on tumour size 43 days after cell inoculation. Note the stimulatory effect of estrogen on the tumour volume which is especially marked in castrated males and females. DHT

FIGURES 3a and 3b

Figure 3a

Growth of human melanomas in estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 control mice.

Note that estradiol treatment slightly increased the growth of the tumours and reduced the delay time; however the tumours in both groups developed at the same rate.

Figure 3b

Growth of human melanomas in estradiol treated male nude mice.

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting estimated tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 control mice.

Note that estradiol stimulated the growth of the tumours in out of cases (see dashed lines).

Figure 3a

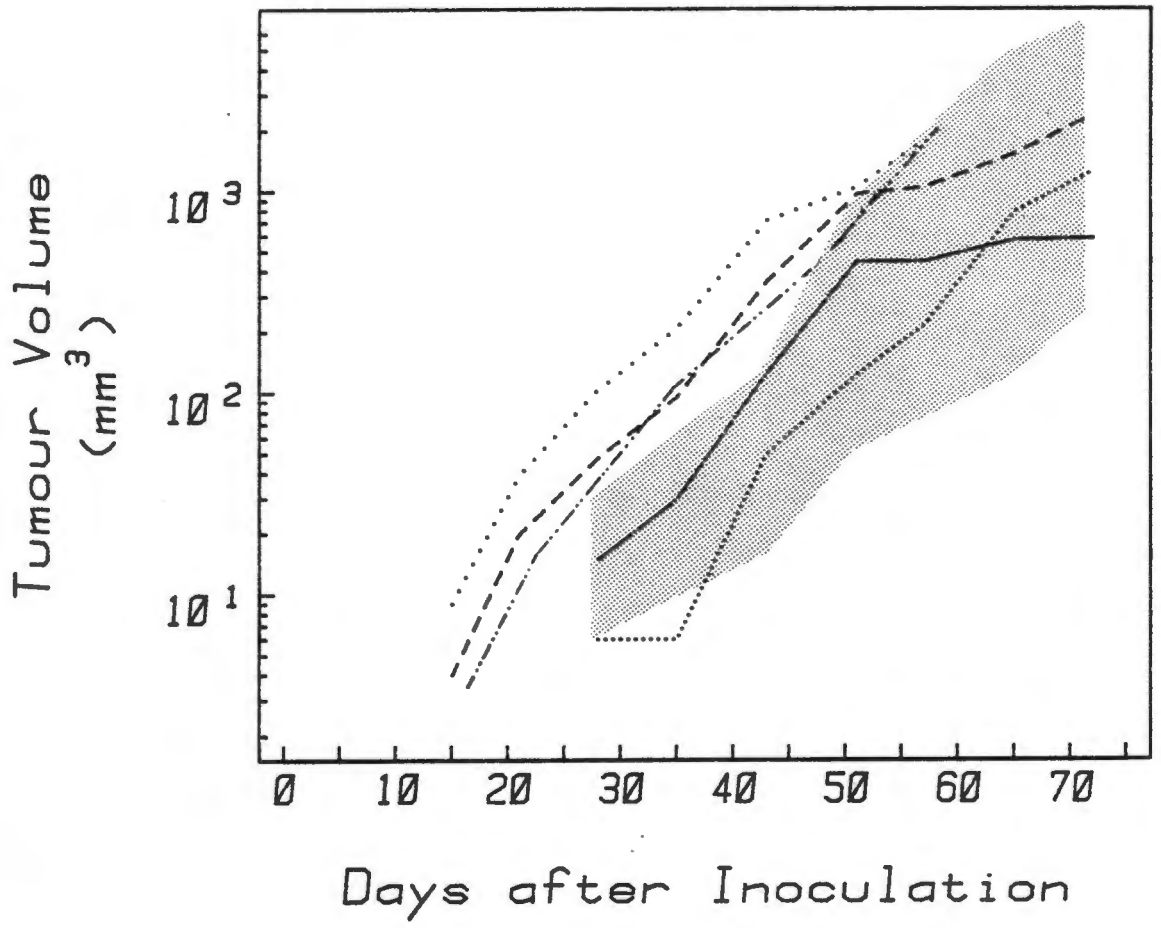
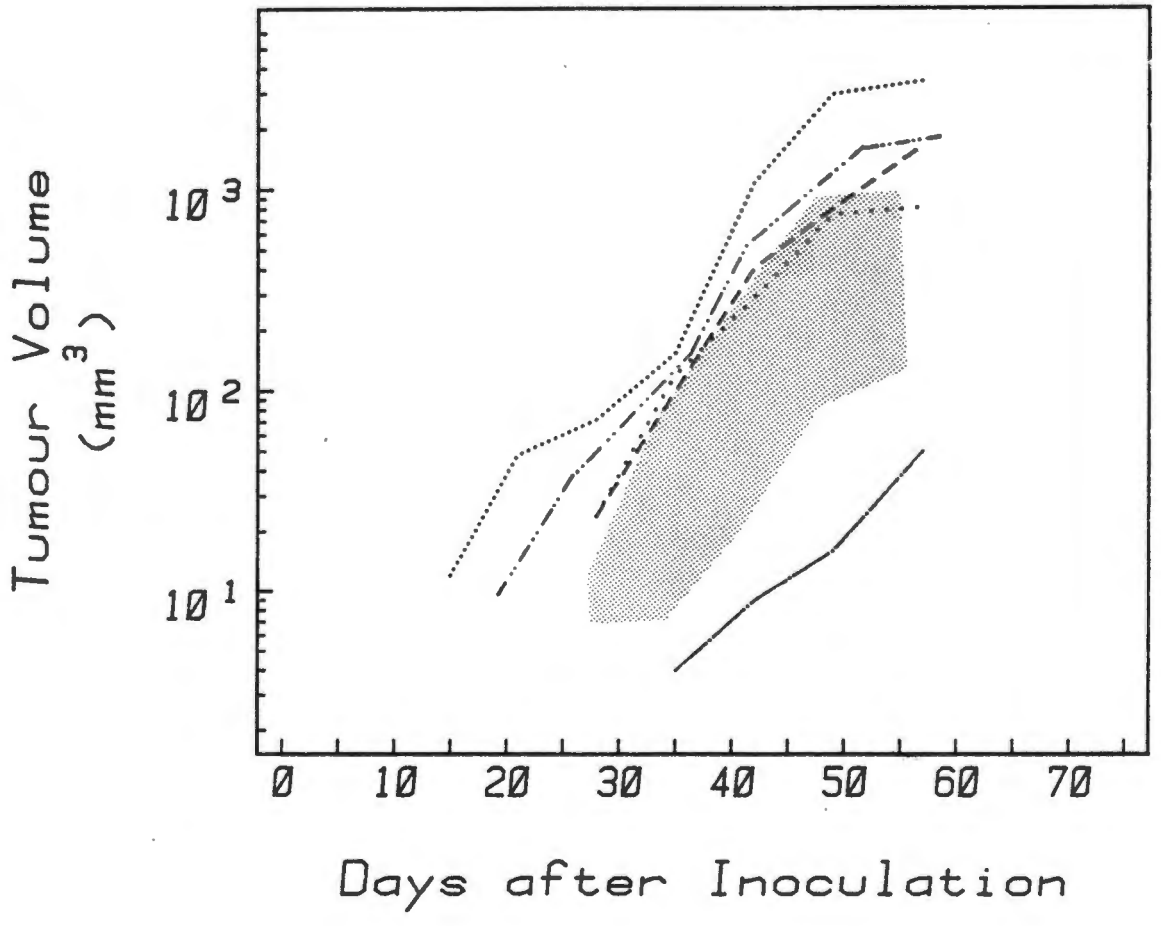


Figure 3b



FIGURES 3c and 3d

Figure 3c

Growth of human melanomas in ovariectomised and estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 3 (69') cells on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 ovariectomised individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 ovariectomised control mice.

Note that in ovariectomised female mice estradiol treatment decreased the delay time of the tumours; however the tumours developed at the same rate.

Figure 3d

Growth of human melanomas in castrated and estradiol treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 castrated individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 3 castrated control mice. The line terminating in an encircled number at the bottom of the figure indicates mice (2) in which tumours failed to develop.

(———) control (2)

Note that as shown previously estradiol decreased the delay time of the tumour.

Figure 3c

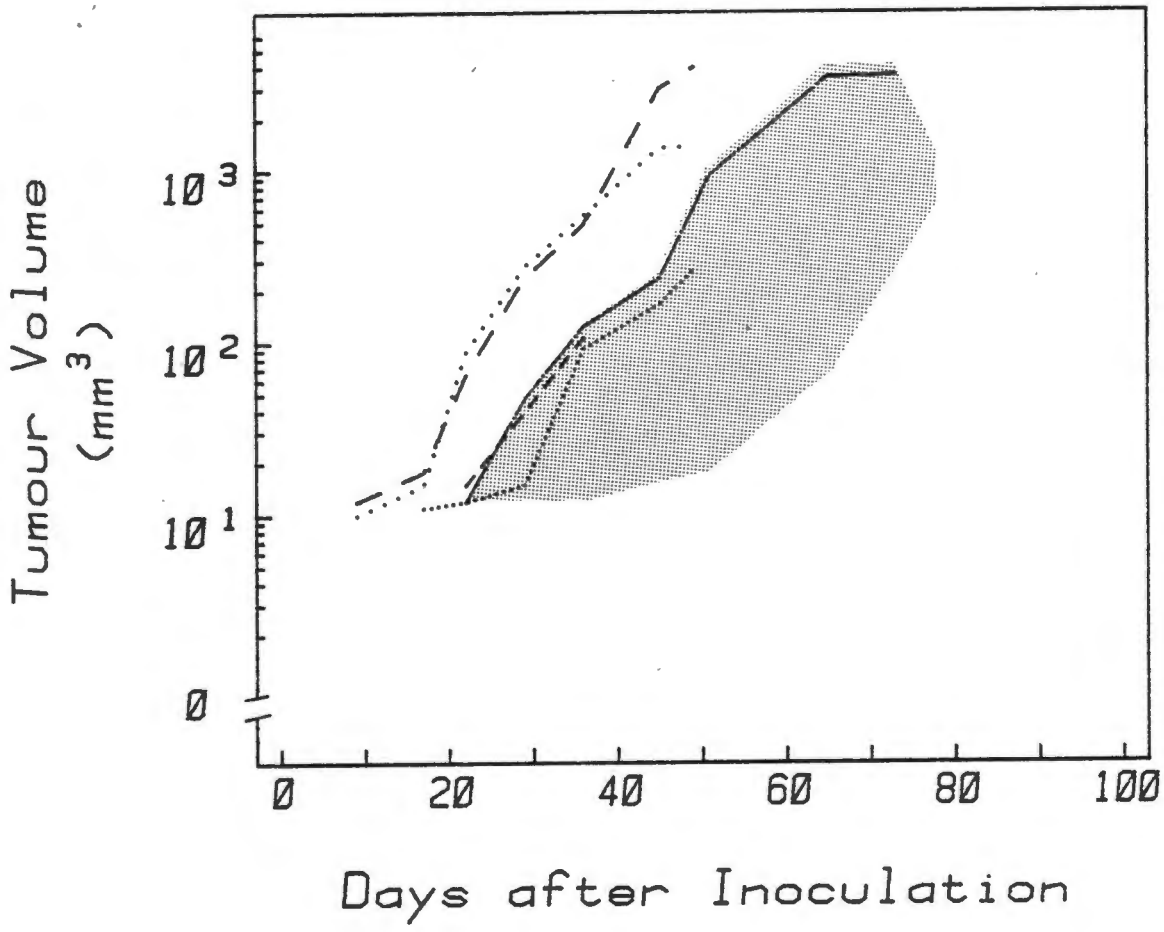


Figure 3d

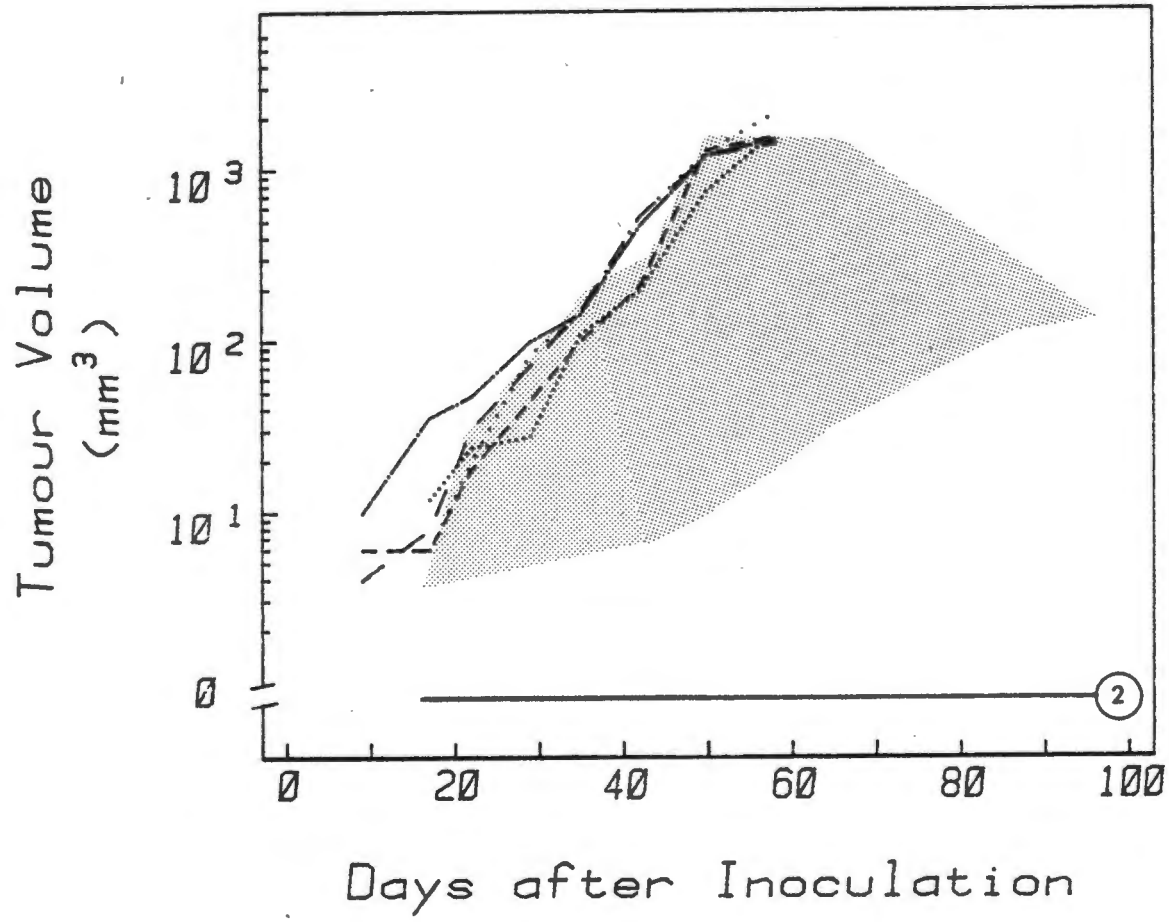


FIGURE 3e

Figure 3e

The effect of castration and hormonal therapy on the growth of UCT-Mel 3 tumours in nude mice.

Male and female mice were castrated and 3-4 weeks later dihydrotestosterone (DHT) or estradiol (E2) pellets were implanted subcutaneously. Twenty-four hours later 1×10^6 UCT-Mel 3 (69') cells were inoculated into castrated and sham-operated animals. Tumour volumes were measured 43 days later. Note the following:-

(a) Castration alone did not result in any alteration in tumour size.

(b) Estradiol resulted in a significant increase in tumour volume especially in the case of ovariectomised female mice. In this instance a 15-fold increase in tumour volume was noted.

▨ - castrated □ - sham-operated

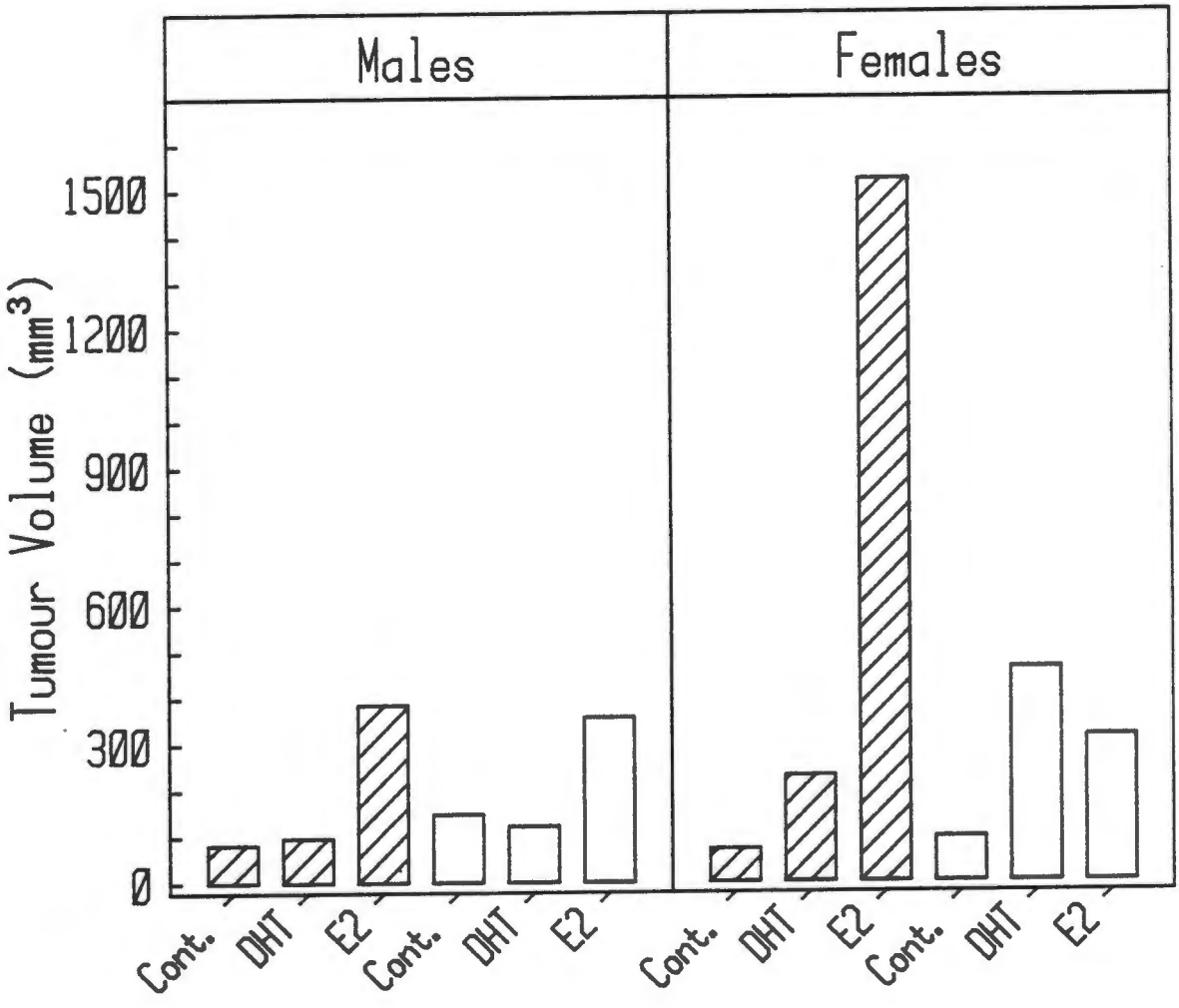

Males
Females
Tumour Volume (mm^3)
1500
1200
900
600
300
0
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2

Figure 3e

FIGURES 3f and 3g

Figure 3f

Growth of human melanomas in ovariectomised and dihydrotestosterone treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual ovariectomised mice treated with dihydrotestosterone (DHT). The shaded area represents the range of tumour volumes for 5 ovariectomised control mice.

Note that DHT treatment decreased the delay time of the tumours in ovariectomised females.

Figure 3g

Growth of human melanomas in dihydrotestosterone treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 2 (124') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with dihydrotestosterone (DHT). The shaded area represents the range of tumour volumes for 5 control mice.

Note that DHT treatment stimulated the growth of the tumours in female mice.

Figure 3f

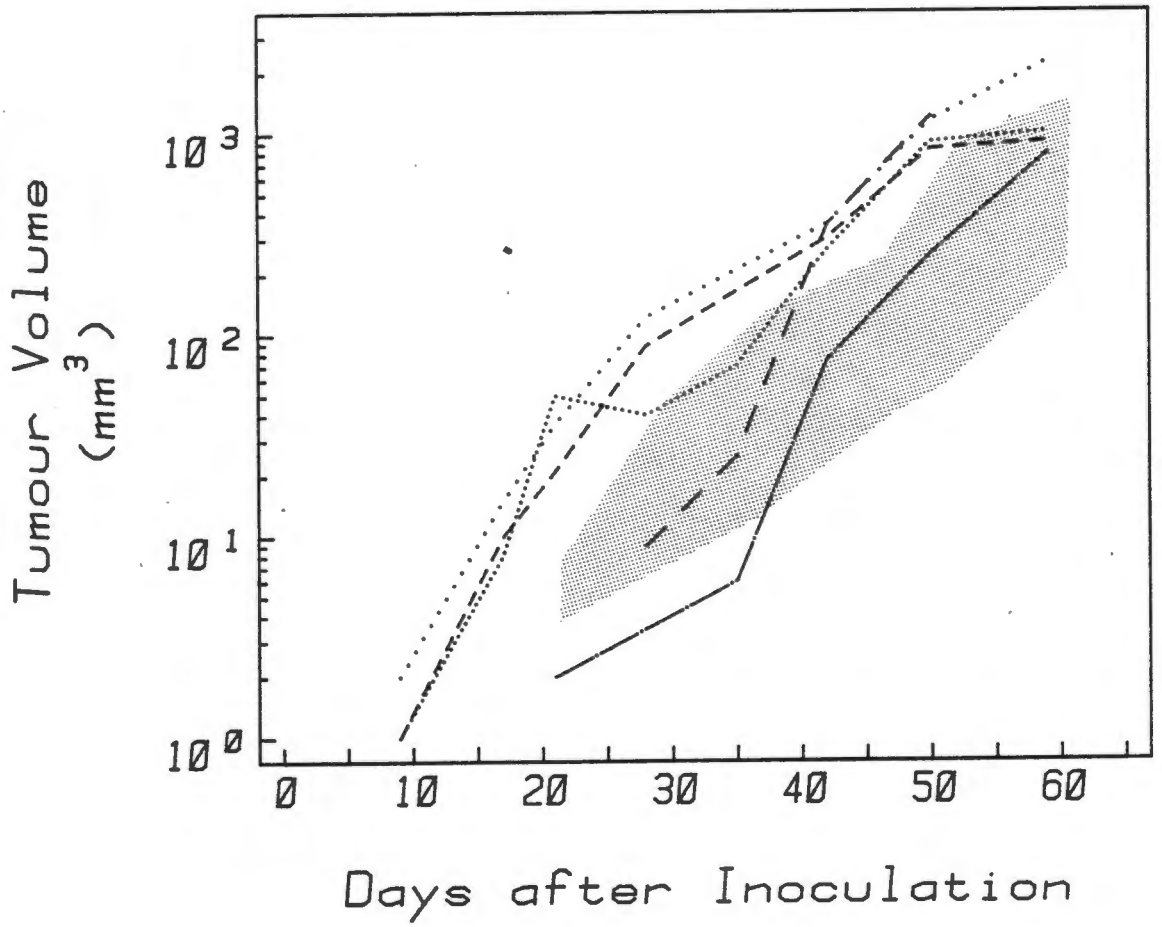
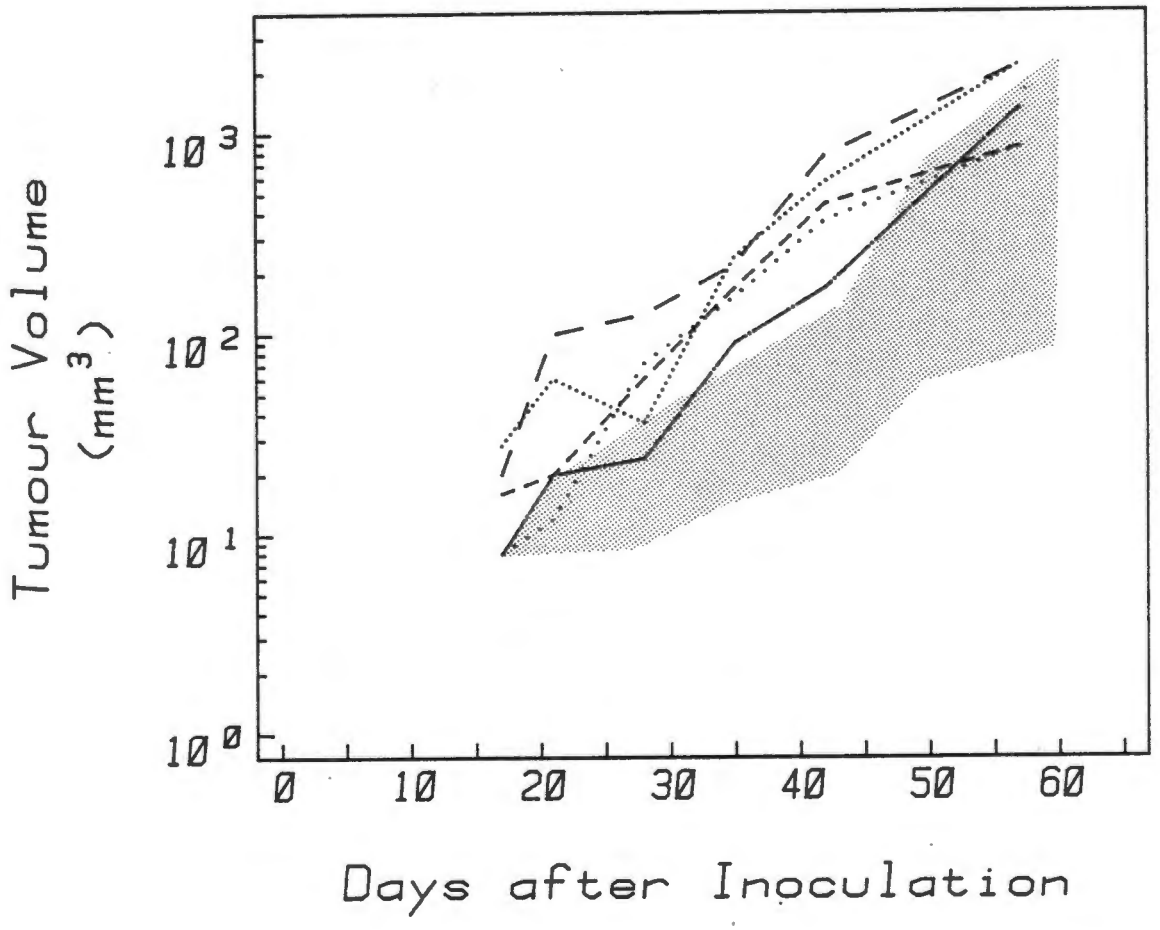


Figure 3g



also had an effect, particularly in female mice where it accelerated the growth of UCT-Mel 3 in both ovariectomized and sham operated females (Figs. 3e, f and g). DHT decreased the T(del)100 from 43 days to 30 days in castrated animals and from 40 days to 29 days in sham operated mice. In castrated male mice the T(del)100 was decreased from 41 to 14 days by DHT.

Experiment (vi)

Table 3.7

Sex	Males						Females					
Operation	Sx			Cx			Sx			Cx		
Treatment	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT
Tumour No.	4/4	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5	6/6	5/5	5/5
T(del)100	–	102	–	–	52	62	–	63	78	–	57	117
Td 200	–	54	–	–	29	25	–	19	43	–	–	–

Mice in 12 groups (Table 3.7) were each inoculated with 10^6 UCT-Mel 7 cells to study the effects of castration, DHT or E2 on tumour cell growth.

This cell line is unusual in as much as it forms tumours that show initial rapid growth after which they reach a plateau in size (cf. Chapter 1, Fig. 1.13). This deviation from the usual Gompertzian kinetics has meant that, in most cases, it has been impossible to derive a Td(200) or a Tdel(100) since the tumours do not usually attain a size of $100mm^3$ or $200mm^3$. If they do get to be $200mm^3$ in size they are usually on the plateau of the growth curve with the result that the Td at $200mm^3$ is infinite.

Sex and/or castration had no effect upon the growth kinetics of UCT-Mel 7 derived tumours (Figs. 4a, b, c and d). E2 supplementation, however, had a striking effect in that some of the tumours in the E2-treated mice grew more

FIGURES 4a and 4b

Figure 4a

Growth of human melanomas in estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 7 cells (30') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 control mice.

Note that estradiol did not affect the growth of the tumours in the first stage of growth, however after 40 days post inoculation the tumour growth increased in females treated with estradiol.

Figure 4b

Growth of human melanomas in ovariectomised and estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 7 cells (30') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual ovariectomised mice treated with estradiol. The shaded area represents the range of tumour volumes for 6 ovariectomised control mice.

Note that estradiol stimulated the growth of tumours in ovariectomised female mice.

Figure 4a

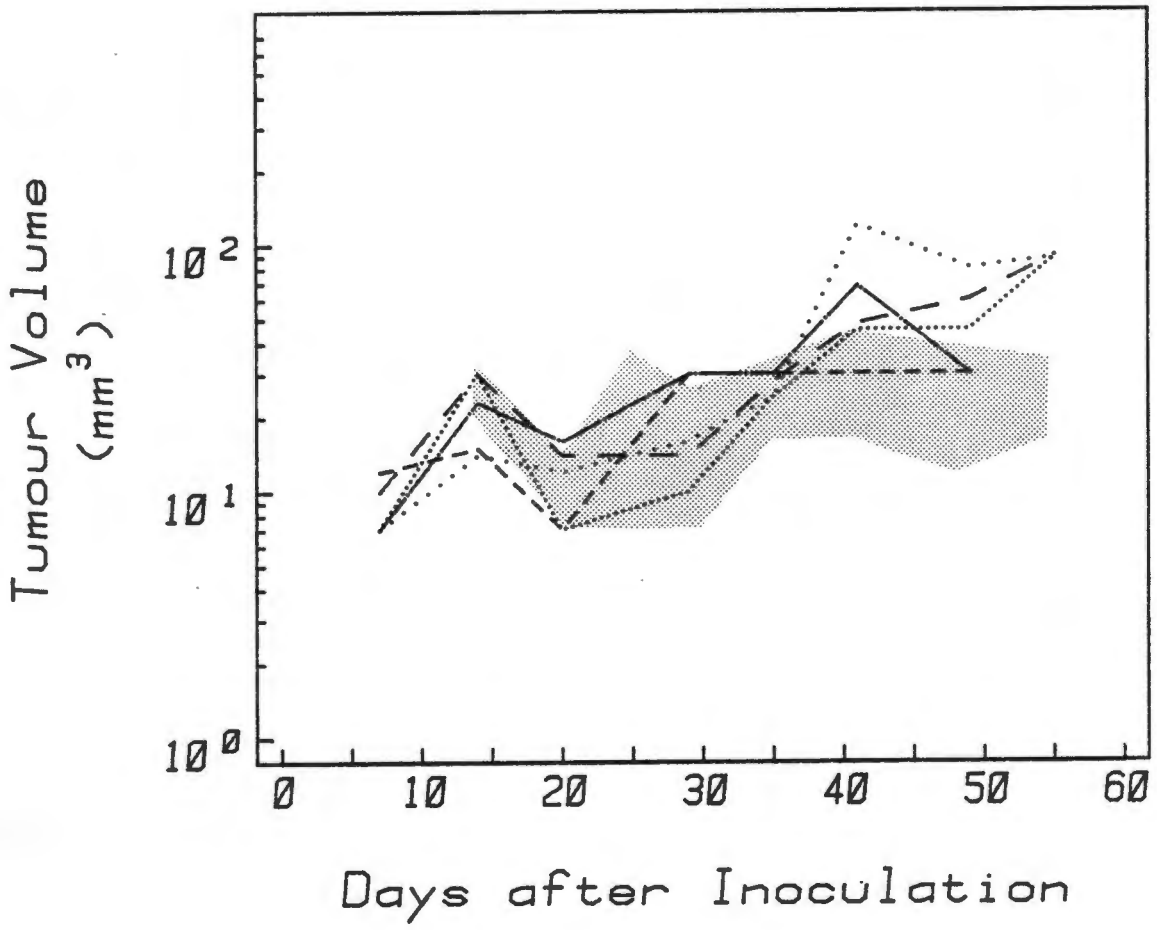
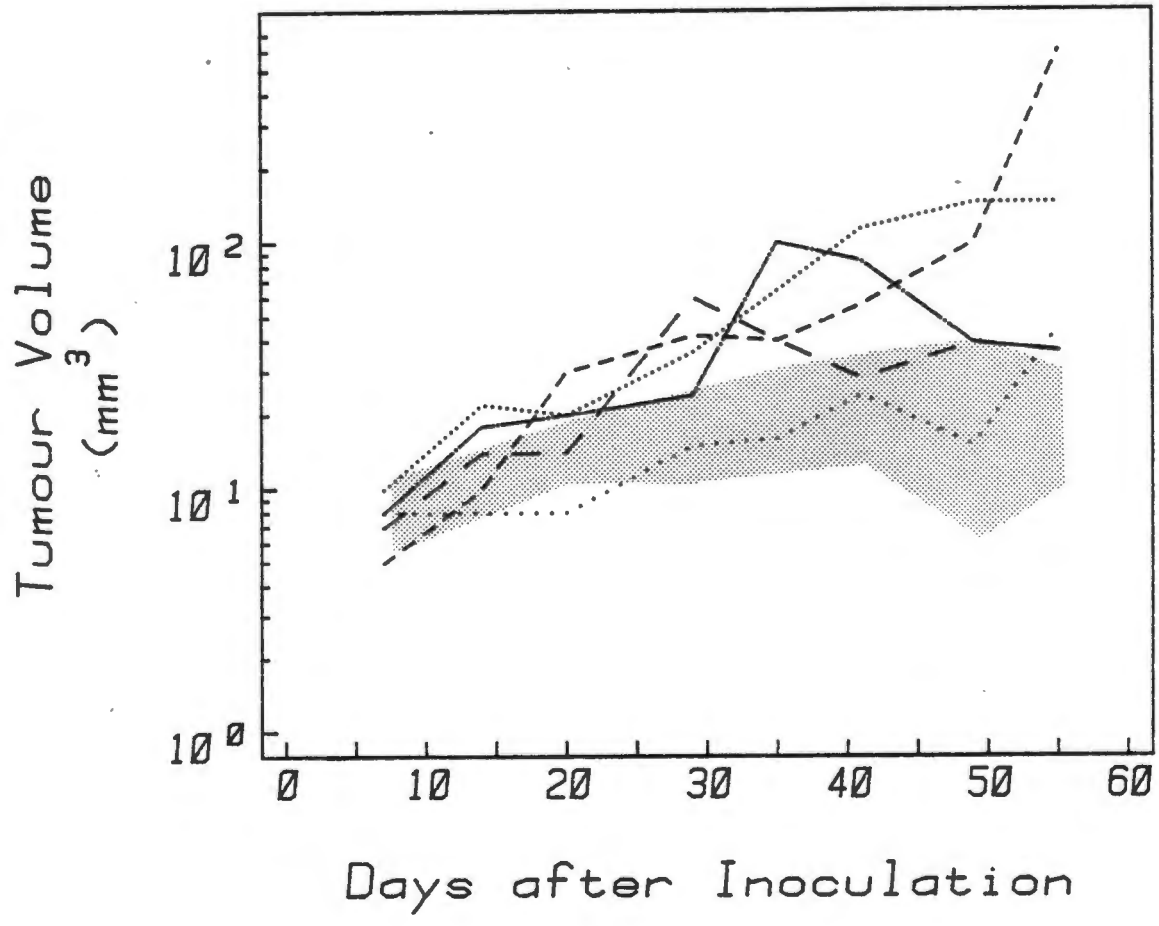


Figure 4b



FIGURES 4c and 4d

Figure 4c

Growth of human melanomas in estradiol treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 7 cells (30') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual male mice treated with estradiol. The shaded area represents the range of tumour volumes for 4 control mice.

Note that estradiol treatment did not alter the growth rate of the tumours in male mice.

Figure 4d

Growth of human melanomas in castrated and estradiol treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 7 cells (30') on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual castrated mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 castrated control mice.

Note that estradiol significantly stimulated the tumour growth in castrated males.

Figure 4c

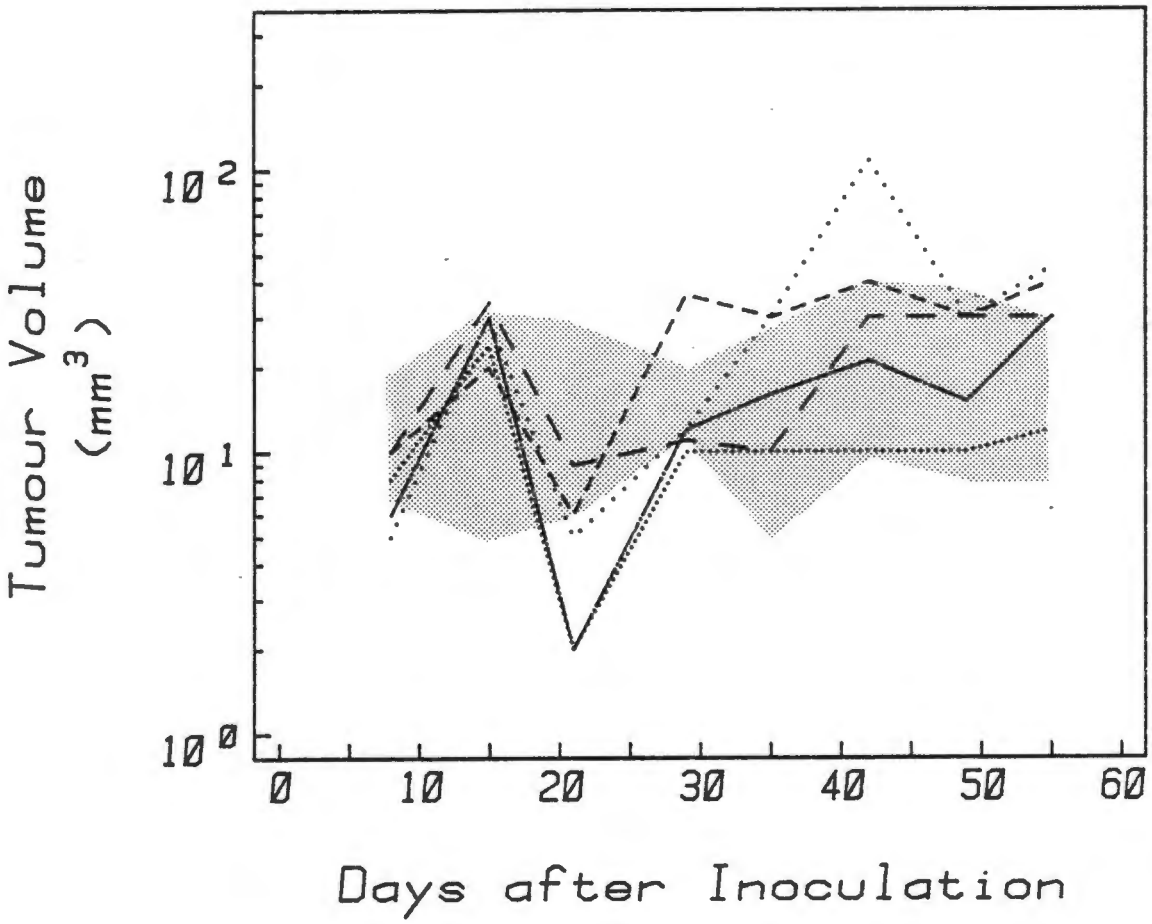
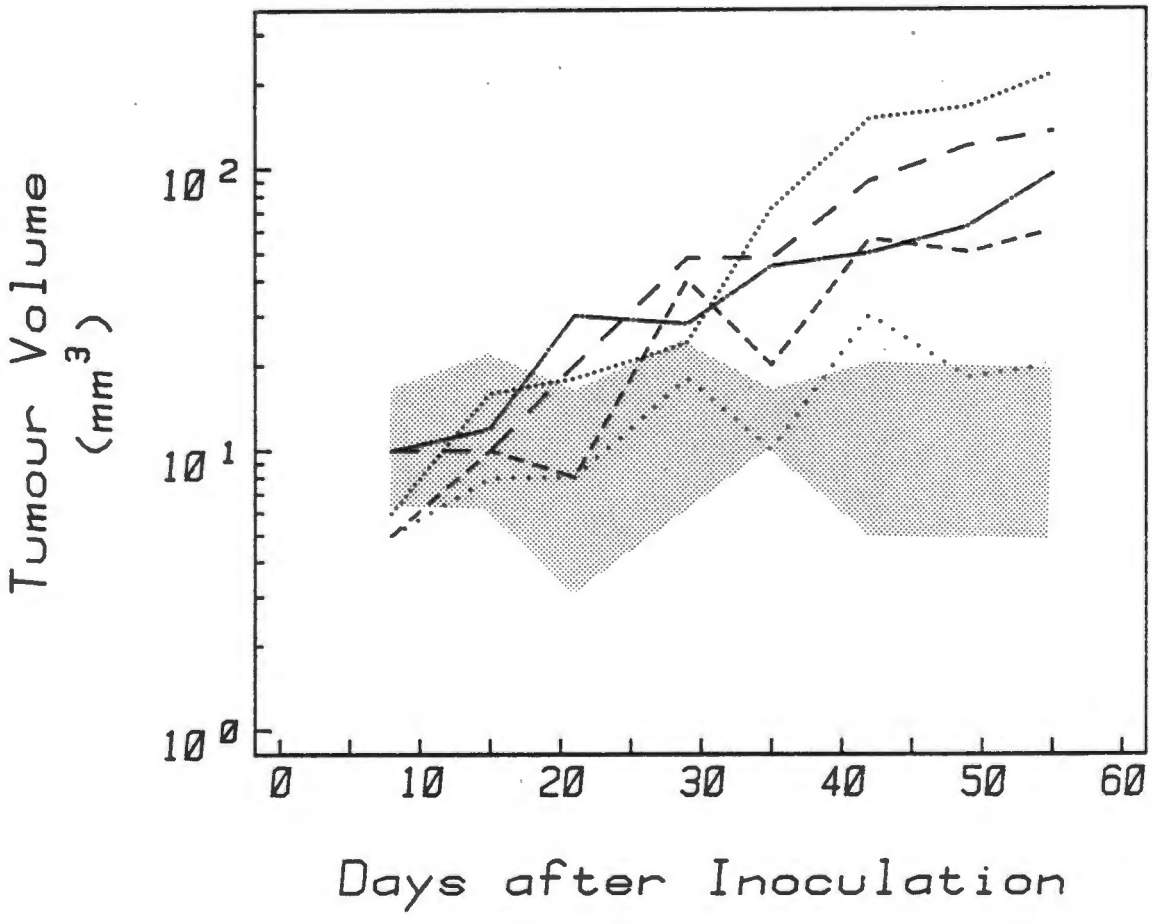


Figure 4d



rapidly and had growth kinetics that resembled those of other tumours (Fig. 4b and 4d). DHT supplementation in normal males had a minimal effect on tumour growth (Fig. 4e) whereas in castrated males had a marked stimulatory effect (Fig. 4f). There was a slight effect of DHT on tumour growth in ovariectomized female mice. These results can also be noted from Fig. 4g which shows the size of UCT-Mel 7 55 days after inoculation of cells. The striking stimulatory effect of E2 and DHT on tumour size in castrated males (see also Fig. 4d and f). and the stimulatory effect of E2 in females (see also Fig. 4b)can be noted.

Experiment (vii)

Table 3.8

Sex	Males						Females					
Operation	Sx			Cx			Sx			Cx		
Treatment	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT	Cnt	E 2	DHT
Tumour No.	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
T(del)100	25	24	28	24	21	28	25	21	23	26	20	29
Td 200	5	4	5	5	5	6	7	4	6	6	5	7

This experiment (Table 3.8) followed the standard protocol described in Experiment (iv) and was designed to study the effect of sex, castration, E2 and DHT on the growth of tumours following inoculation of 10^6 UCT-Mel 1 cells.

The cells used in this experiment were a subline of UCT-Mel 1 which had been adapted to grow under serum-free conditions.

The tumours that resulted grew rapidly in male mice (Fig. 5b)and E2 had little effect in this group. In females, however, there was a distinct tendency for the tumours to appear and develop more slowly than in males. E2 administration expedited the appearance of tumours in females so that growth

FIGURES 4e and 4f

Figure 4e

Growth of human melanomas in dihydrotestosterone treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 7 cells (30') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with dihydrotestosterone (DHT). The shaded area represents the range of tumour volumes for 4 control mice.

Note that DHT treatment had no effect on the growth of tumours in male mice.

Figure 4f

Growth of human melanomas in castrated and dihydrotestosterone treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 7 cells (30') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual castrated mice treated with dihydrotestosterone (DHT). The shaded area represents the range of tumour volumes for 5 castrated control mice.

Note that DHT treatment stimulated the tumour growth in castrated mice.

Figure 4e

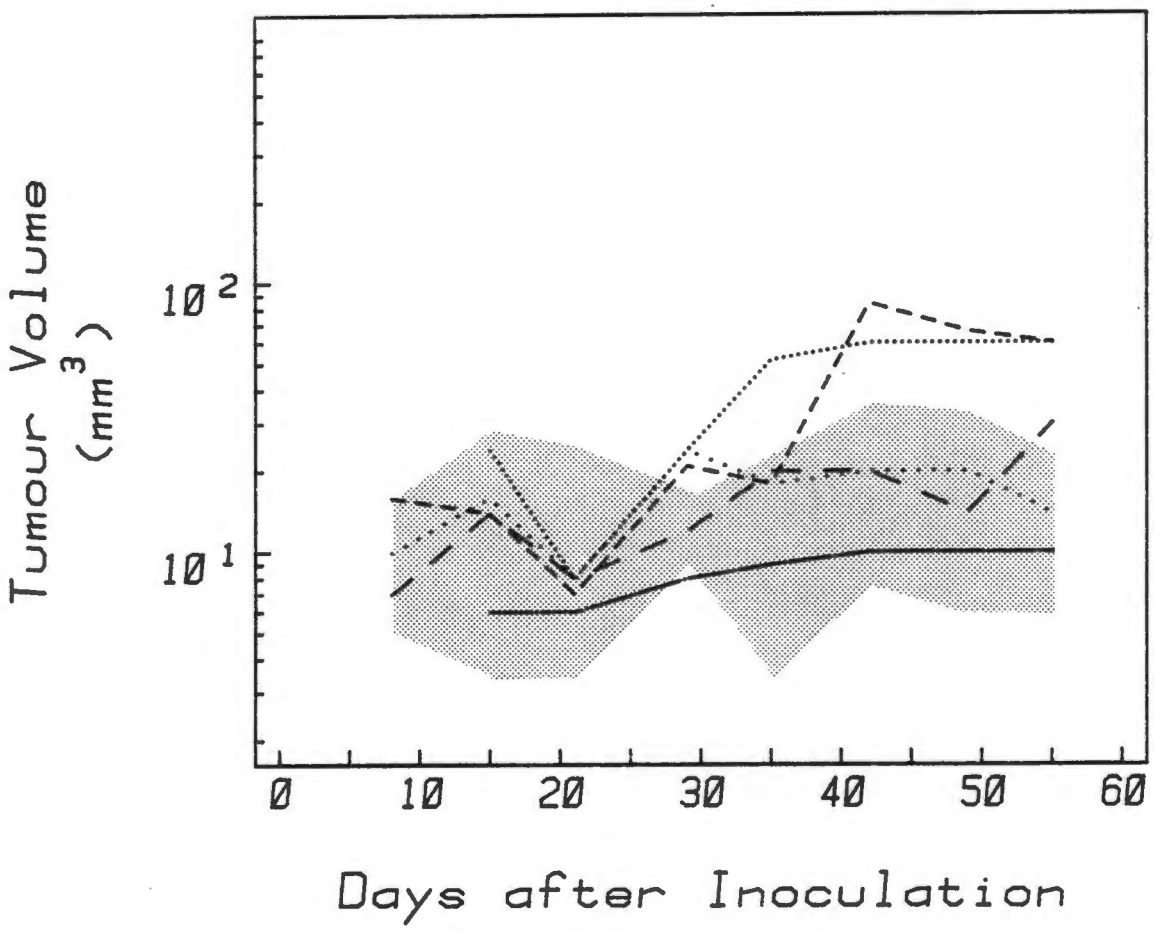


Figure 4f

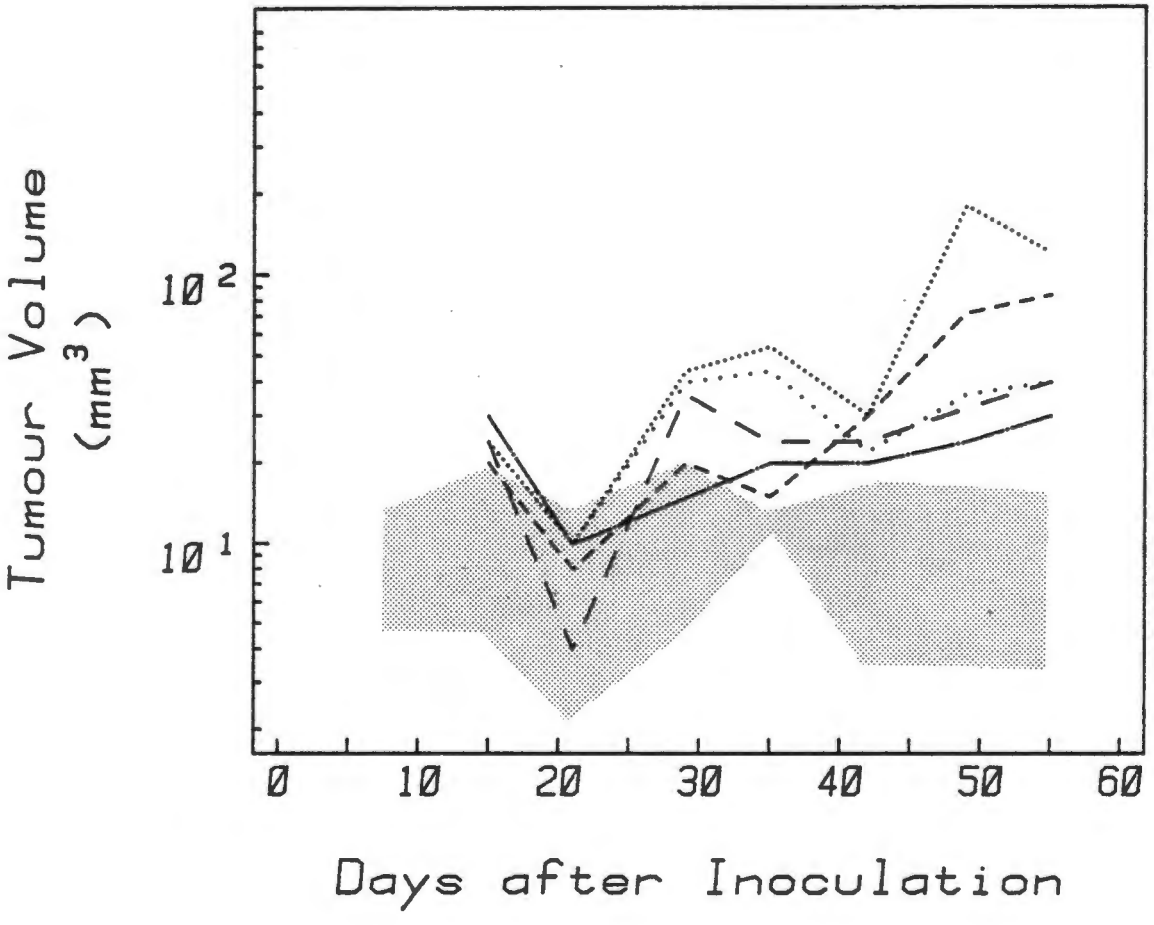


FIGURE 4g

Figure 4g

The effect of castration and hormonal therapy on the growth of UCT-Mel 7 tumours in nude mice.

Male and female mice were castrated and 3-4 weeks later dihydrotestosterone (DHT) or estradiol (E2) pellets were implanted subcutaneously. Twenty four hours later 1×10^6 UCT-Mel 7 cells (30') were inoculated into castrated and sham-operated animals. Tumour volume was measured 55 days later. Note the following:-

(a) Both DHT and estradiol stimulated growth of tumours in castrated male and female mice.

(b) Estradiol markedly stimulated the growth of tumours in sham-operated female mice.

 - castrated  - sham-operated

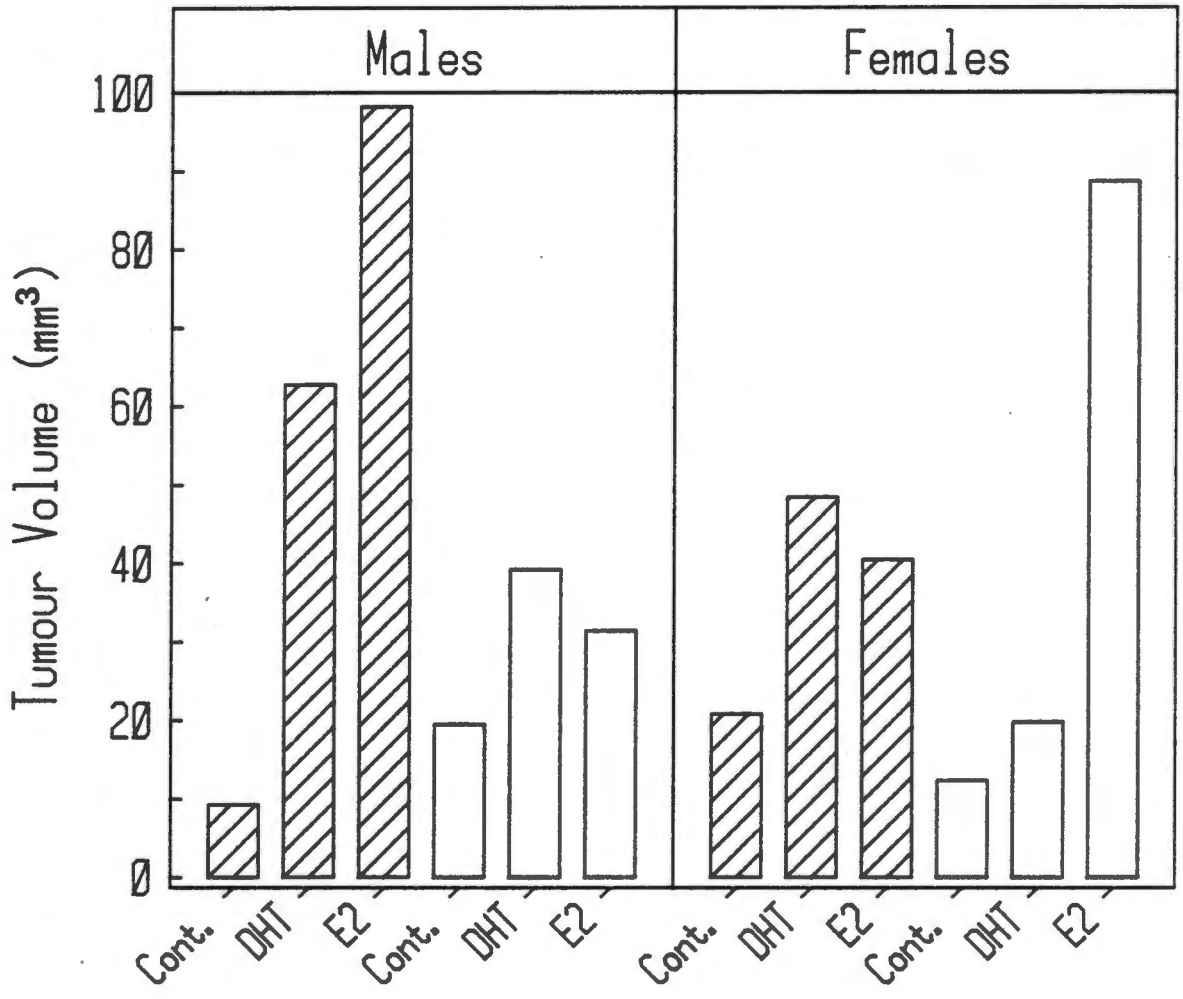

Males
Females
Tumour Volume (mm3)
100
80
60
40
20
0
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2

Figure 4g

FIGURES 5a and 5b

Figure 5a

Growth of human melanomas in estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 1 cells (serum-free line) on day 0 and tumour volumes determined at the indicated times. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 control mice.

Note that estradiol stimulated the tumour growth in female mice.

Figure 5b

Growth of human melanomas in estradiol treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 1 cells (serum-free line) on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 control mice.

Note that estradiol treatment had no effect on the growth of tumours in male mice.

Figure 5a

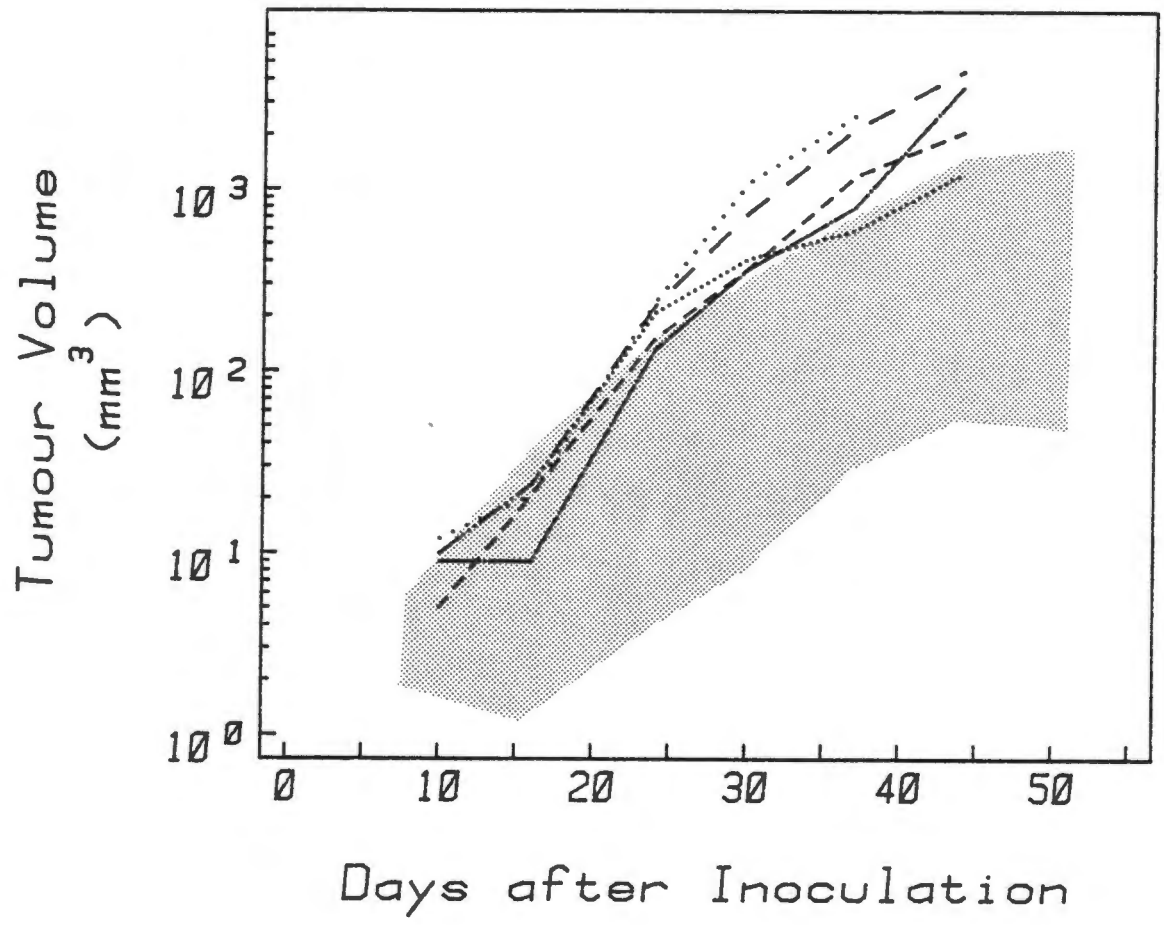
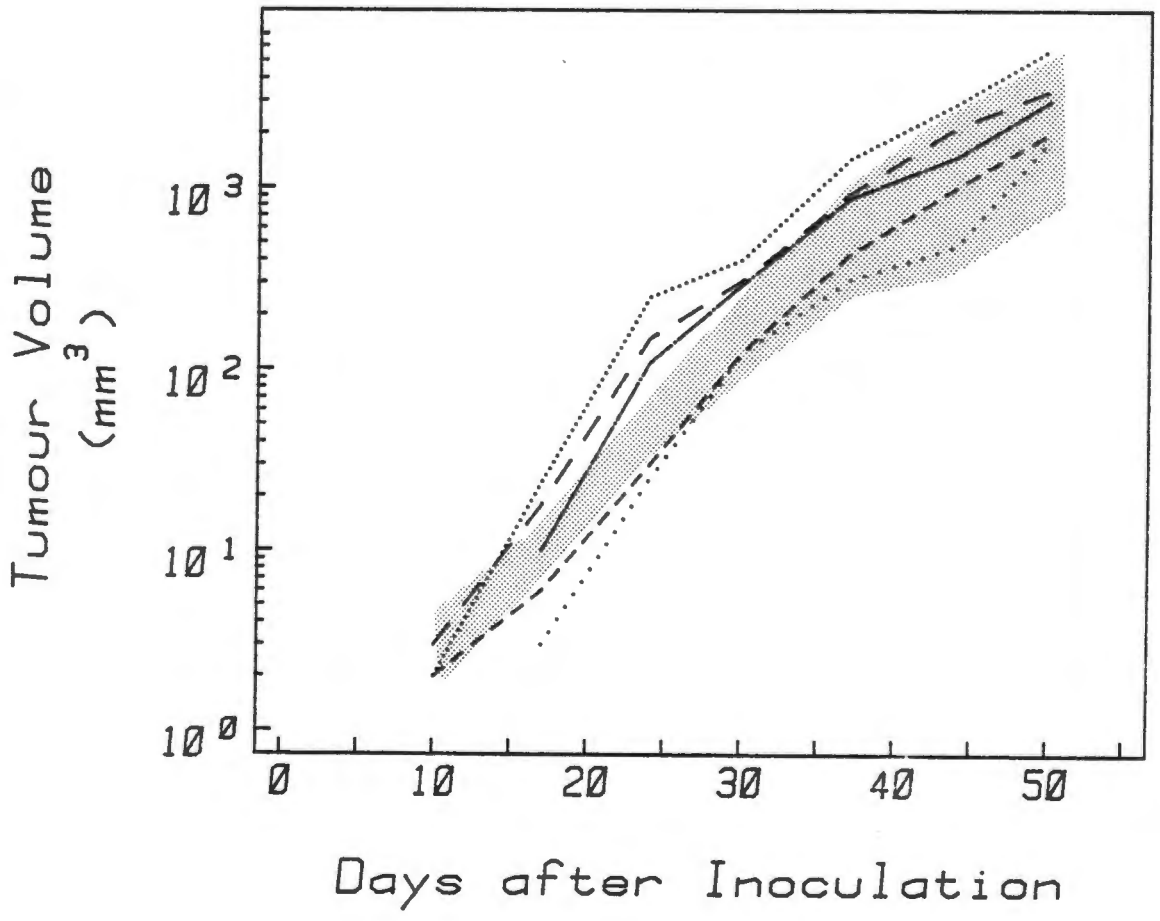


Figure 5b



kinetics in females resembled those in males (Fig. 5a).

DHT inhibited tumour growth in castrated and sham-operated male mice (Figs. 5c and d) but had no effect on tumour development in female mice.

These results are further summarized in Fig. 5e which shows the tumour volume of UCT-Mel 1 cells 44 days after cell inoculation. By this time E2 treated ovariectomized and sham-operated female mice had larger tumours than untreated controls. In contrast DHT inhibited tumour growth in castrated and sham-operated male mice.

Experiment (viii)

Table 3.9

Sex	Females									
Operation	Sx					Cx				
Treatment	Cnt	E 2	DHT	TAM E 2	DHT E 2	Cnt	E 2	DHT	TAM E 2	DHT E 2
Tumour No.	4/5	5/5	5/5	5/5	5/5	4/5	5/5	5/5	5/5	4/5
T(del)100	32	34	35	27	32	38	32	35	26	41
Td 200	6	5	7	6	6	6	6	7	6	16

Since E2 appeared to have an effect upon the growth of tumours derived from UCT-Mel 3 cells (Experiment (v)) it was of interest to note the effect of the anti-estrogenic compound, tamoxifen, on the development of tumours when this was given with E2 to sham-operated or castrated females. An experiment was accordingly performed in which ten groups of female mice were castrated or sham-operated and treated with DHT, E2, DHT + E2, or tamoxifen + E2 as shown in the table 3.9.

E2, as observed previously, stimulated tumour growth in castrated animals (Fig. 6a) and in this experiment had a minimal effect in control mice (Fig. 6b). However in both these groups of animals, tamoxifen and E2 given together had a pronounced stimulatory effect on tumour growth (Figs. 6c, d and e).

FIGURES 5c and 5d

Figure 5c

Growth of human melanomas in castrated and dihydrotestosterone treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 1 (serum-free line) on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual castrated mice treated with dihydrotestosterone (DHT). The shaded area represents the range of tumour volumes for 5 castrated untreated mice.

Note that DHT treatment slightly reduced the size of the tumours in castrated male mice.

Figure 5d

Growth of human melanomas in dihydrotesterone treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 1 (serum-free line) on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual mice treated with dihydrotestosterone (DHT). The shaded area represents the range of tumour volumes for 5 control mice.

Note that DHT treatment, as shown previously, decreased the size of the tumours in male mice.

Figure 5c

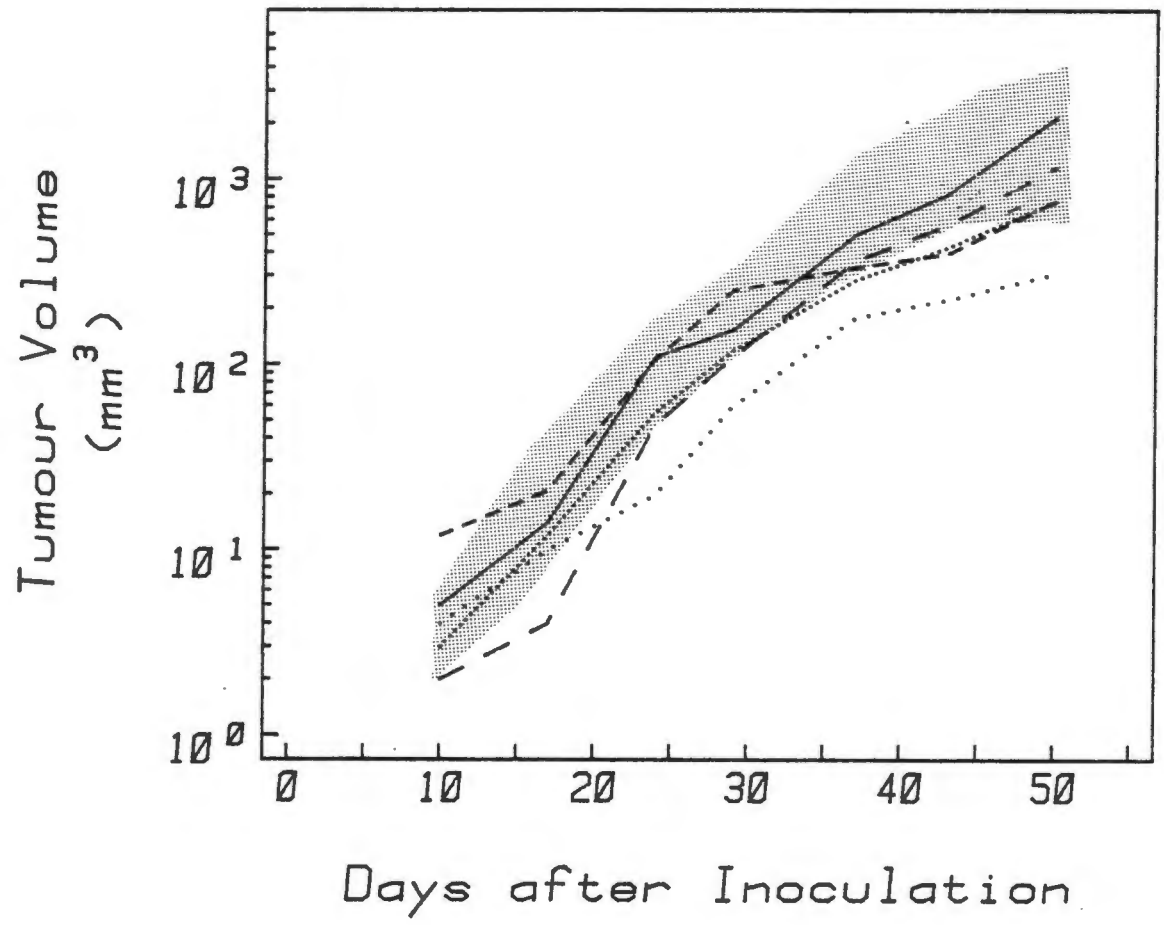


Figure 5d

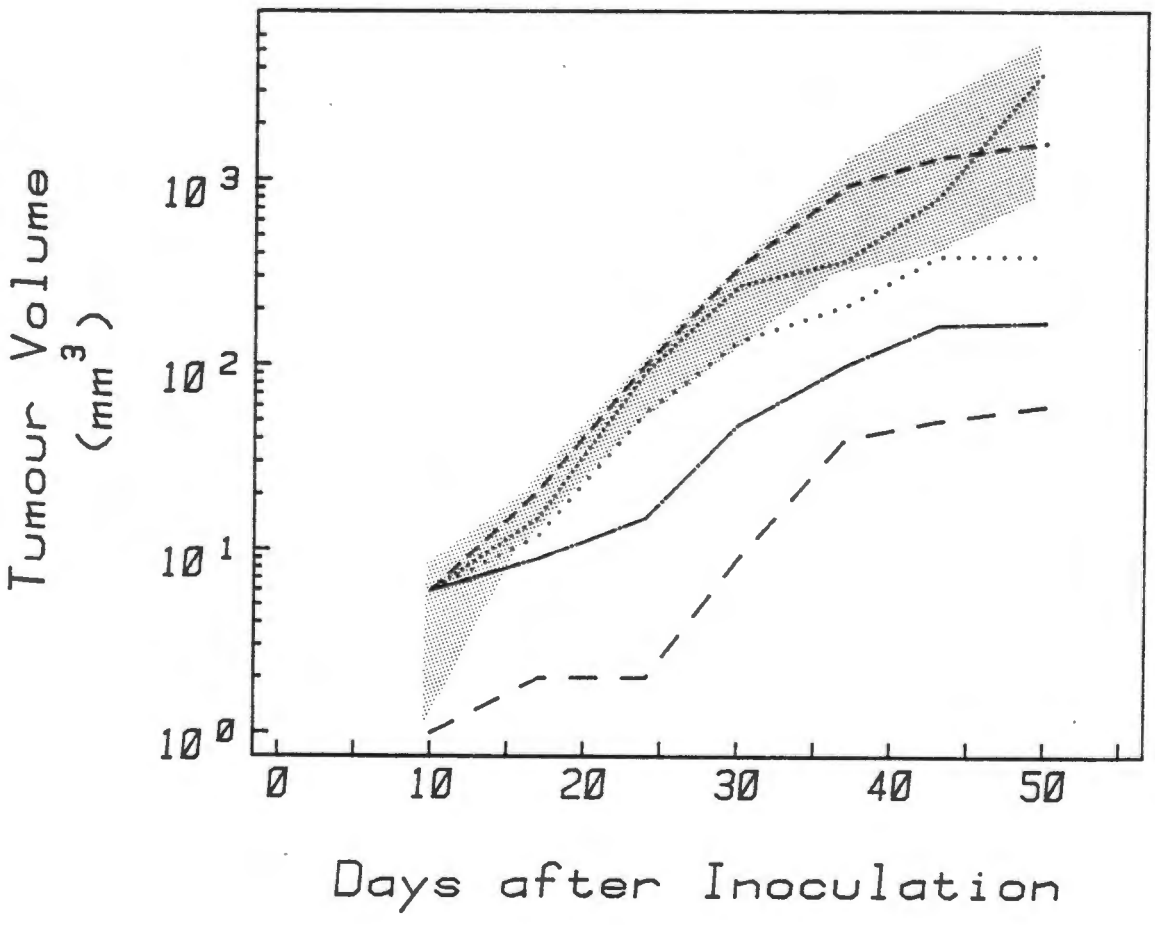


FIGURE 5e

Figure 5e

The effect of castration and hormonal therapy on the growth of UCT-Mel 1 tumours in nude mice.

Male and female mice were castrated and 3-4 weeks later dihydrotestosterone (DHT) or estradiol (E2) pellets were implanted subcutaneously. Twenty-four hours later 10^6 UCT-Mel 1 cells (serum-free line) were inoculated into castrated and sham-operated animals. Tumour volume was measured 44 days later.

Note the following:-

(a) Castration alone did not result in any alteration in tumour size.

(b) DHT suppressed the growth of tumours in both intact and castrated male mice.

(c) Estradiol treatment stimulated tumour growth in ovariectomised and sham-operated female mice.

▨ - castrated ☐ - sham-operated

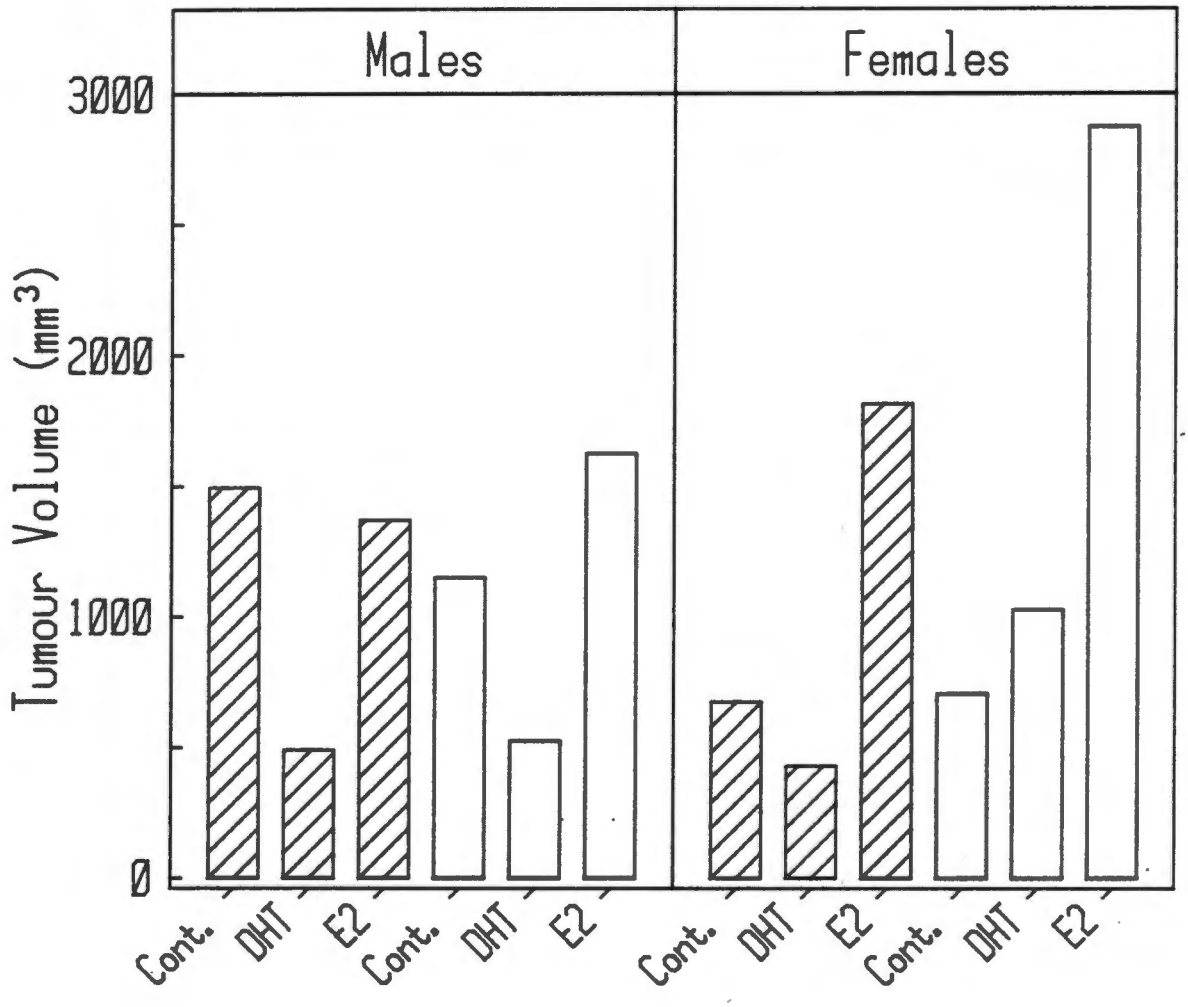

Males
Females
3000
2000
1000
0
Tumour Volume (mm3)
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2
Cont.
DHT
E2

Figure 5e

FIGURES 6a and 6b

Figure 6a

Growth of human melanomas in ovariectomised and estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual ovariectomised mice treated with estradiol. The shaded area represents the range of tumour volumes for 4 ovariectomised control mice. The line terminating in an encircled number at the bottom of the figure indicates the control mouse (1) in which tumours failed to develop.

(————) control (1)

Note that estradiol treatment stimulated the growth of tumours in 4 of 5 cases (see dashed lines).

Figure 6b

Growth of human melanomas in estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 4 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 4 control mice. The line terminating in an encircled number at the bottom of the figure indicates the control mouse (1) in which tumours failed to develop.

(————) control (1)

Note that estradiol treatment had no effect on the growth of tumours in intact female mice.

Figure 6a

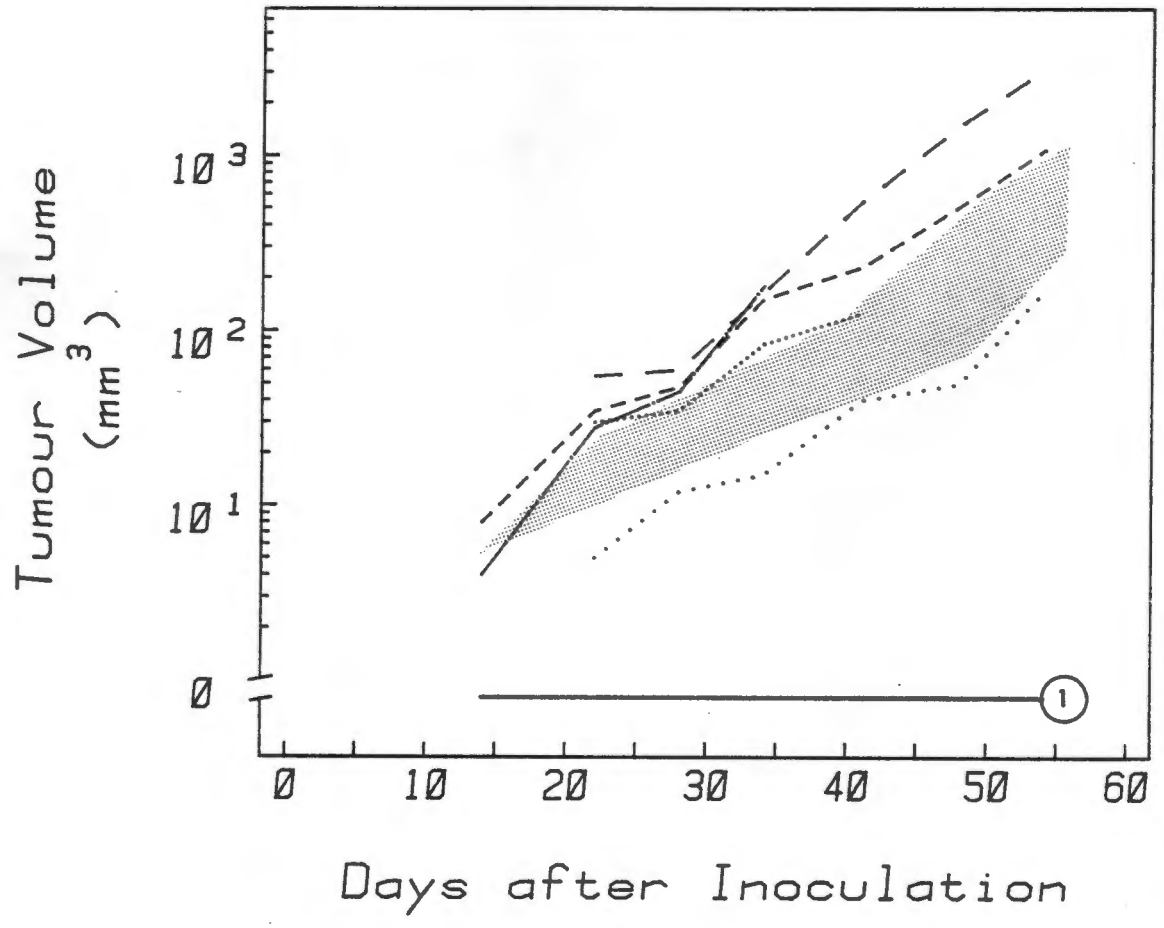
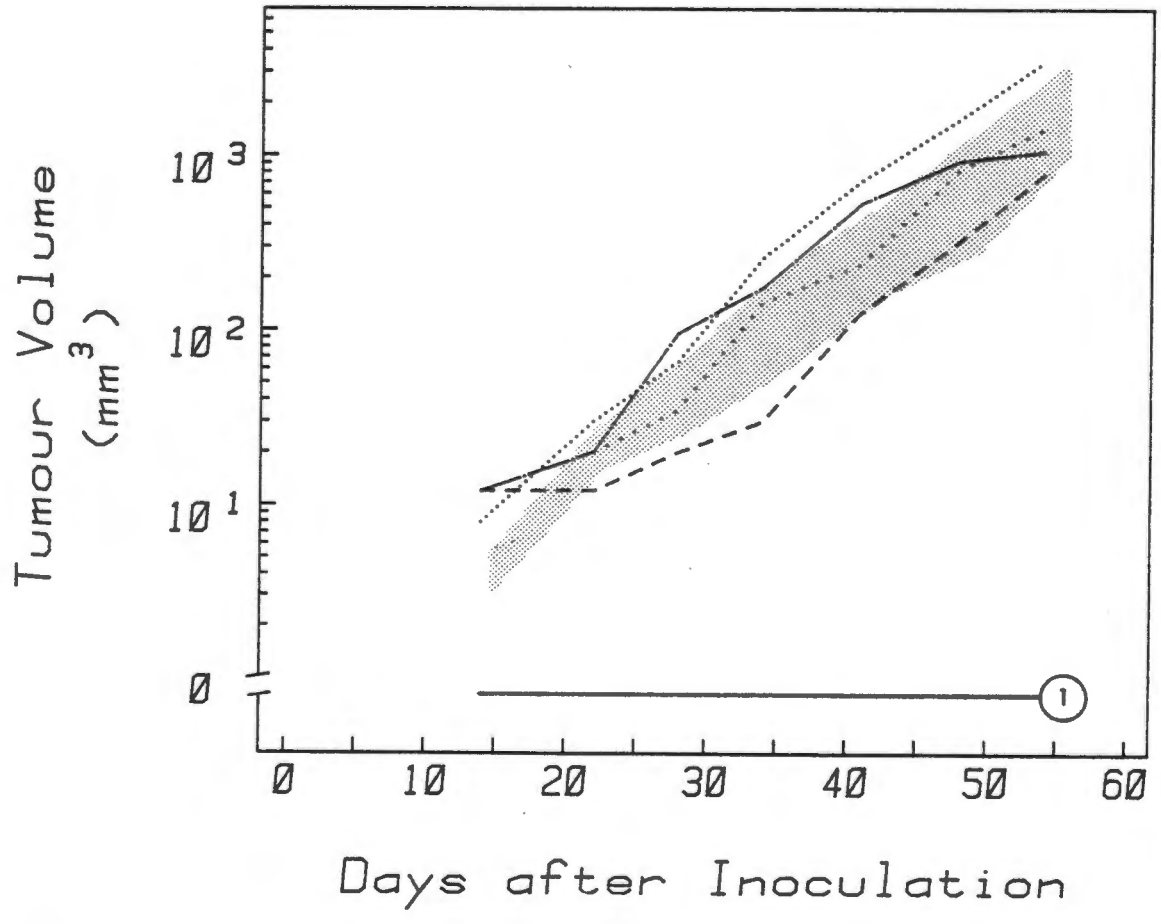


Figure 6b



FIGURES 6c and 6d

Figure 6c

Growth of human melanomas in ovariectomised and hormonally treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on Day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 ovariectomised individual mice treated with estradiol and tamoxifen (E2 + TAM). The shaded area represents the range of tumour volumes for 4 ovariectomised mice treated with estradiol.

The line terminating in an encircled number at the bottom of the figure indicates the control ovariectomised mouse (1) in which tumour failed to develop.

Note that TAM and E2 together decreased the delay time and stimulated tumour growth in ovariectomised female mice.

(———) control (1)

Figure 6d

Growth of human melanomas in hormonally treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 3 cells (69') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual mice treated with estradiol and tamoxifen (E2 + TAM). The shaded area represents the range of tumour volumes for 4 untreated control mice. The line terminating in an encircled number at the bottom of the figure indicates the control untreated mouse (1) in which tumours failed to develop.

(———) estradiol (1)

TAM and E2 decreased the delay time and stimulated tumour growth.

Figure 6c

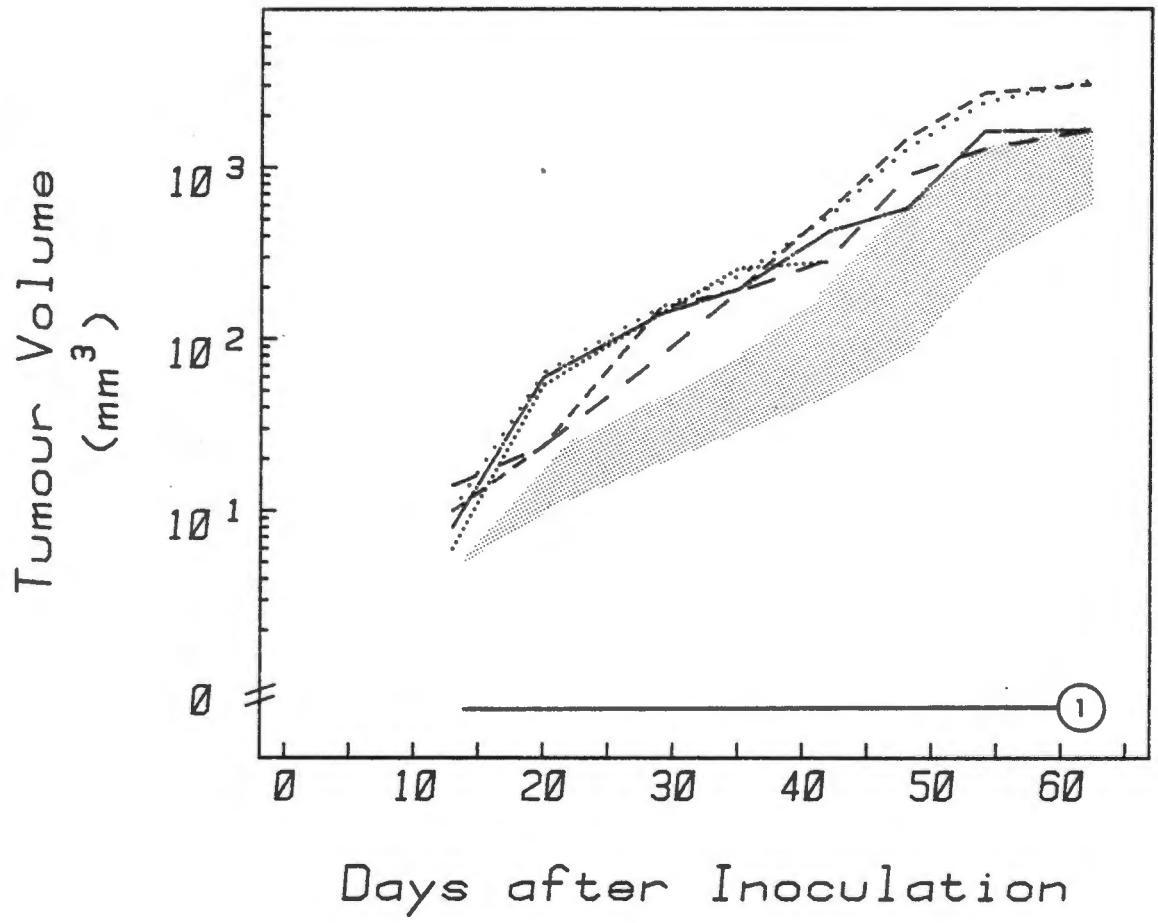


Figure 6d

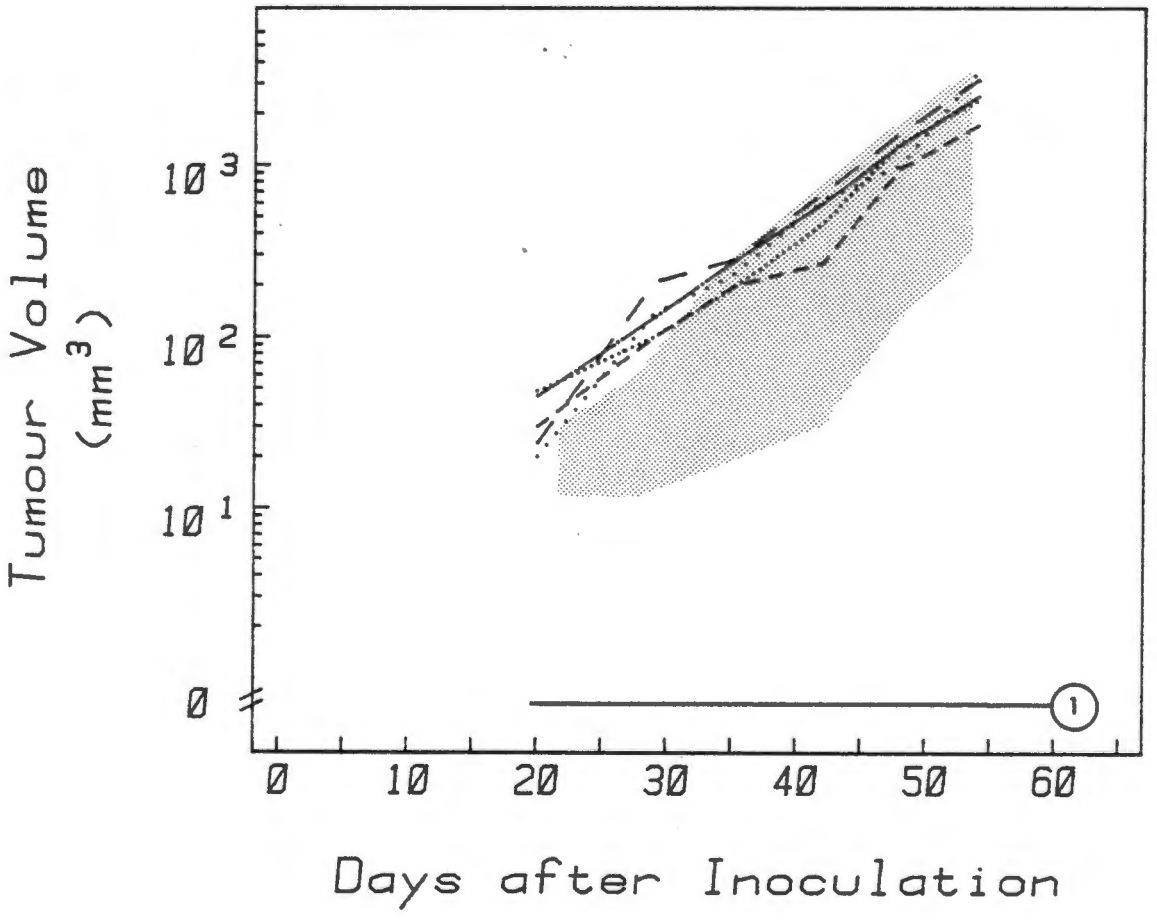


FIGURE 6e

Figure 6e

The effect of ovariectomy and hormonal therapy on the growth of UCT-Mel 3 tumours in female nude mice.

Female mice were ovariectomised and 3-4 weeks later dihydrotestosterone (DHT), estradiol (E2), tamoxifen and E2 (TAM + E2) or DHT and E2 (DHT + E2) pellets were implanted subcutaneously. Twenty four hours later 10^6 UCT-Mel 3 cells (69') were inoculated into ovariectomised and sham-operated animals. Tumour volume was determined 41 days later.

Note the following:-

(a) Castration alone decreased tumour size in female mice. Growth was restored by E2 administration.

(b) The stimulatory effect of estradiol was further enhanced by tamoxifen therapy in ovariectomised animals.

(c) Tamoxifen + E2 stimulated tumour growth in sham operated animals.

 - castrated

 -sham-operated

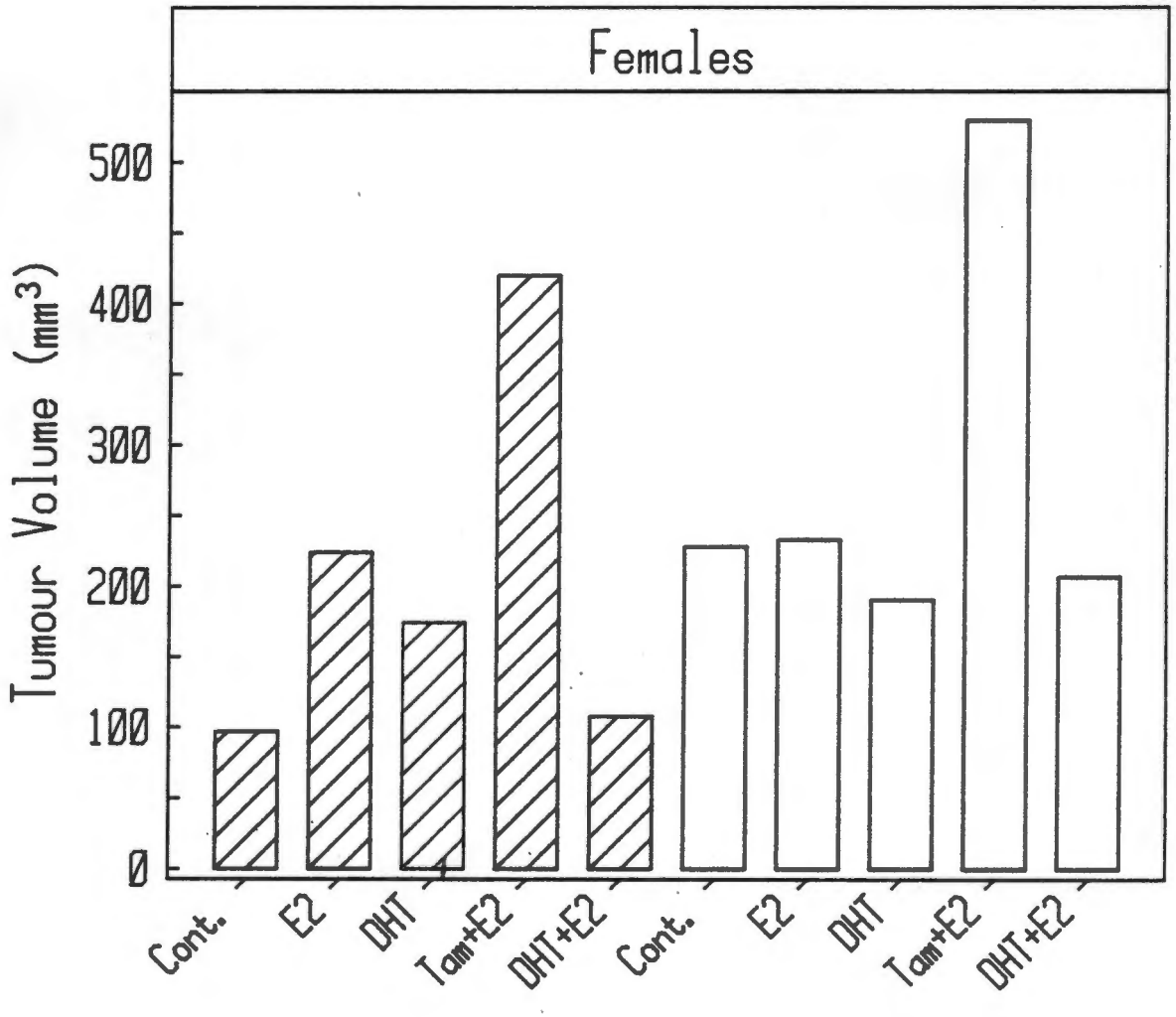

Females
Tumour Volume (mm3)
500
400
300
200
100
0
Cont.
E2
DHT
Tam+E2
DHT+E2
Cont.
E2
DHT
Tam+E2
DHT+E2

Figure 6e

The latent period in castrated animals treated with E2 and tamoxifen was also reduced to 26 days from 38 days (Table 3.9). DHT alone or in combination with E2 had no significant effect on tumour growth.

Experiment (ix)

Table 3.10

Sex	Males		Females					
Treatment	Cnt	E 2	Cnt	E 2	TAM E 2	TAM	PRO	PRO E 2
Tumour No.	3/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5
T(del)100	20	21	19	20	16	20	20	20
Td 200	10	5	6	5	4	5	6	6

This experiment (Table 3.10) was designed to study the effects of E2, tamoxifen and progesterone (PRO), either alone or in combination, on the growth of tumours from inocula of 10^6 UCT-Mel 1 cells. The results showed that as found previously E2 stimulated the growth of tumours (Figs. 7a and 7b). As noted with UCT-Mel 3 tumour growth (Fig. 6c and 6d) tamoxifen and E2 given together resulted in a still further increase in tumour size (Figs. 7c and 7d). Tamoxifen and progesterone alone and progesterone together with E2 had no effect on tumour growth (Fig. 7d).

FIGURES 7a and 7b

Figure 7a

Growth of human melanomas in estradiol treated male nude mice

Male mice were inoculated subcutaneously with 10^6 UCT-Mel 1 (serum-free line) on day 0 and the tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 3 control mice. The line terminating in an encircled number at the bottom of the figure indicates control mice (2) in which tumours failed to develop.

(————) control (2)

Note that estradiol therapy increased tumorigenicity in male mice.

Figure 7b

Growth of human melanomas in estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 1 cells (serum-free line) on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes of 4 control mice. The line terminating in an encircled number at the bottom of the figure indicates a control mouse (1) in which tumours failed to develop.

(————) control (1)

Note that estradiol therapy had no effect on the growth of tumours in female mice; however tumorigenicity was slightly increased in estradiol treated mice.

Figure 7a

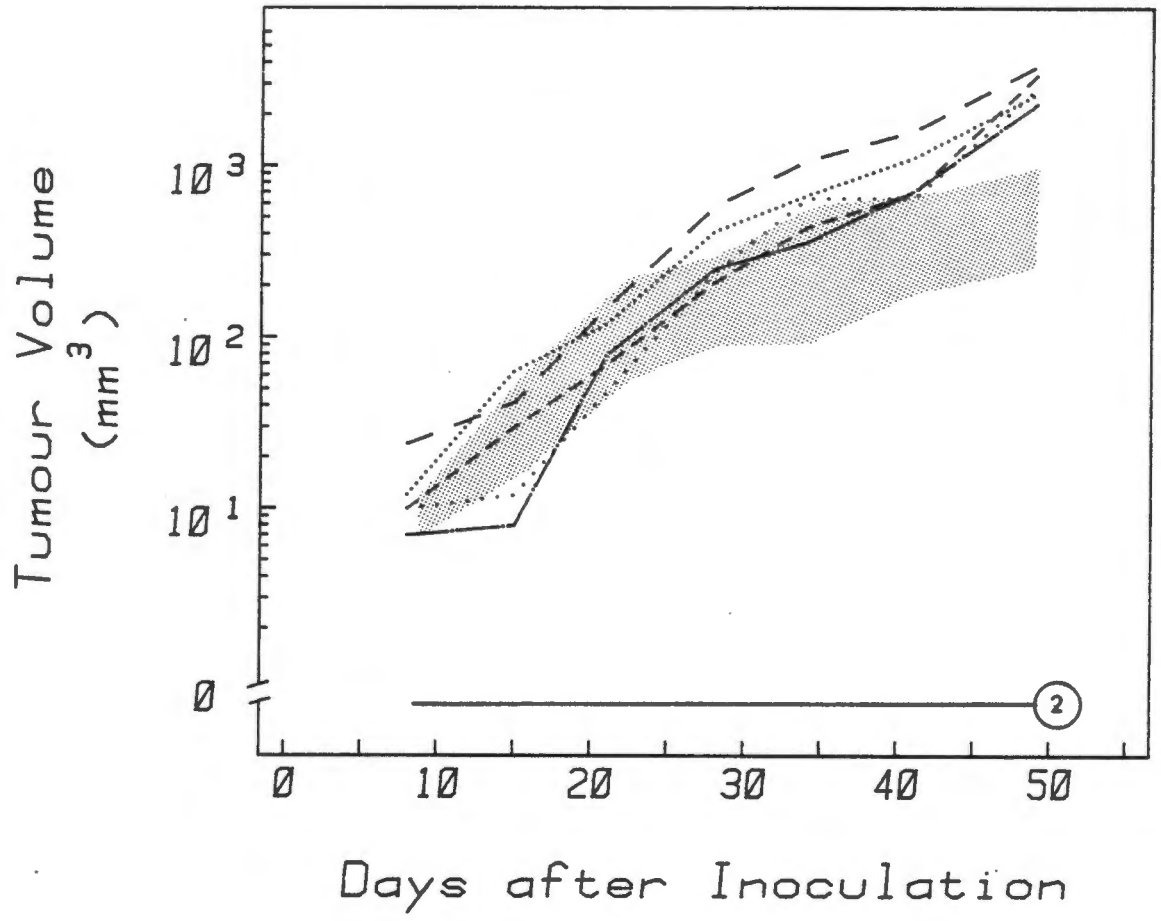
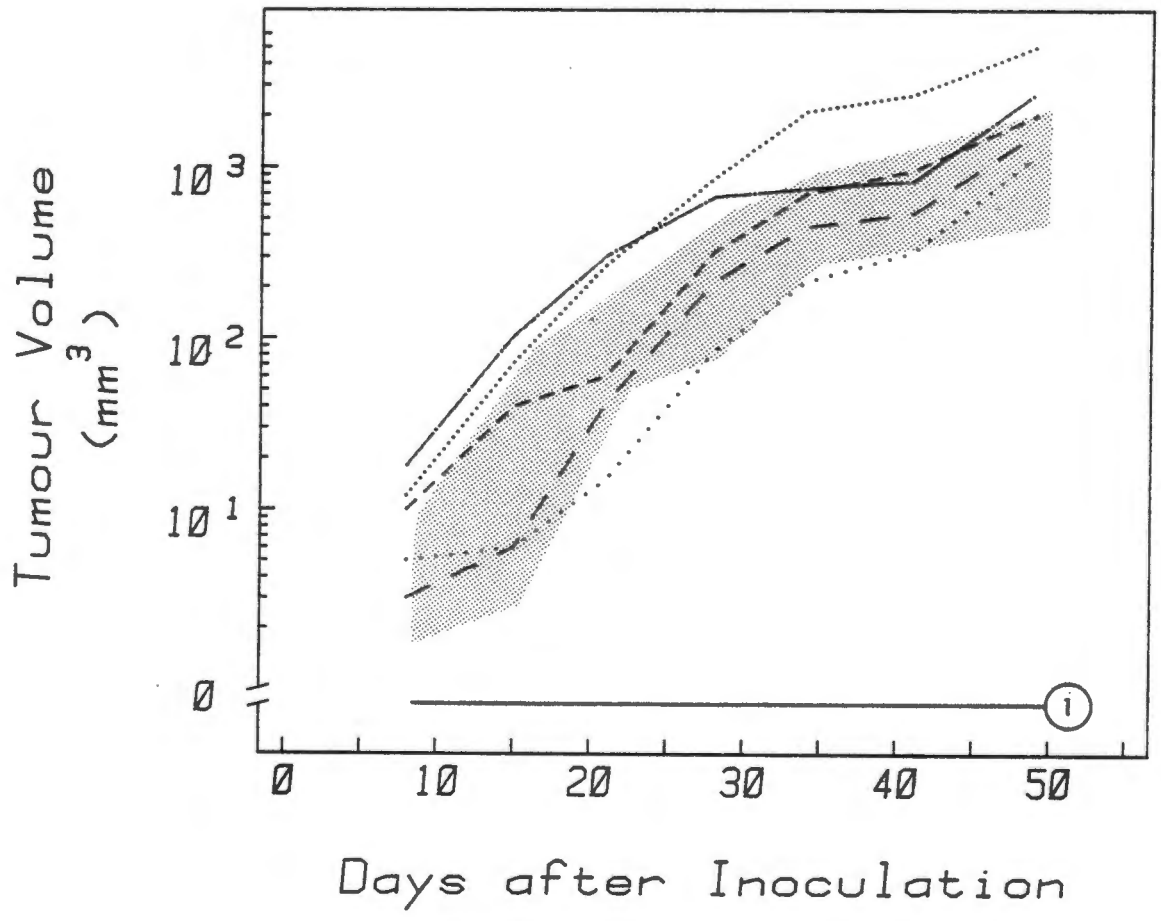


Figure 7b



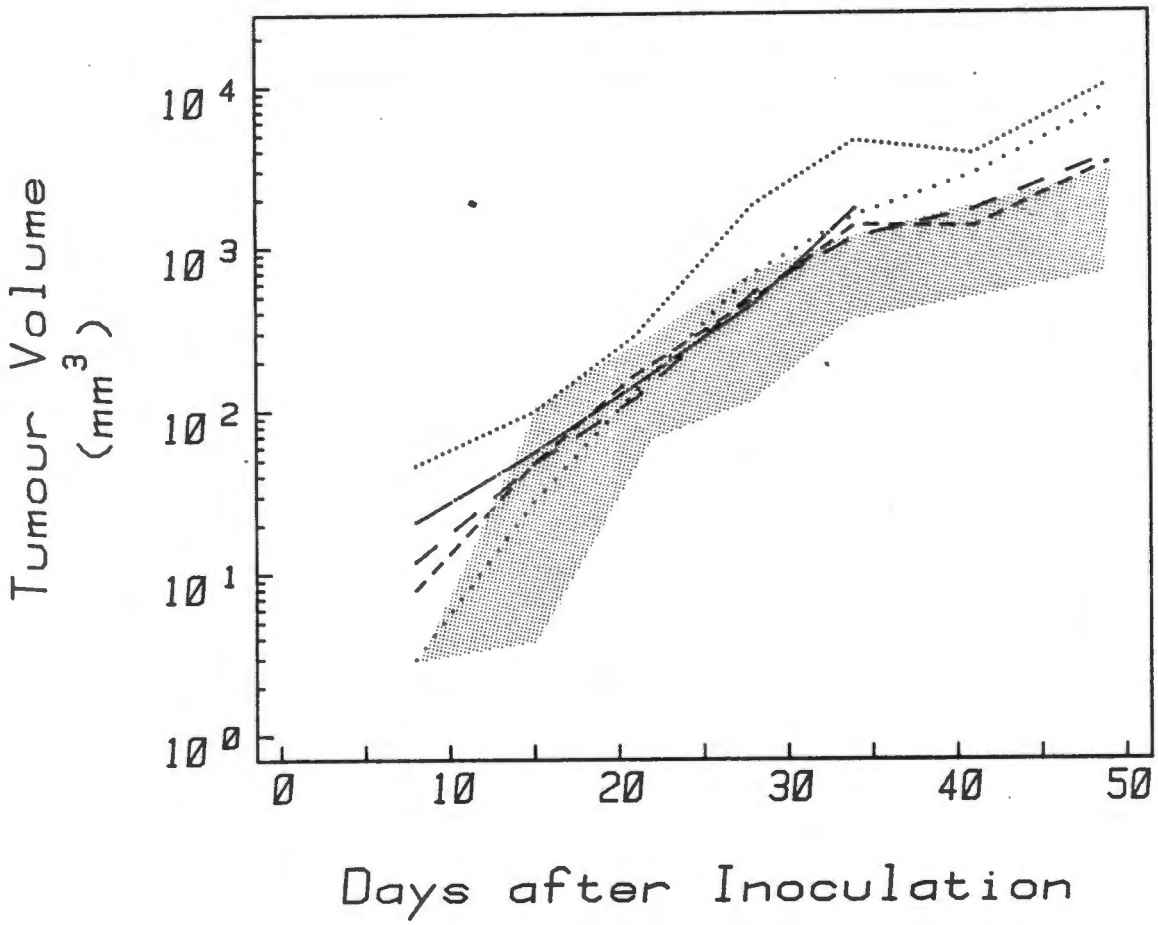


Figure 7c

Growth of human melanomas in hormonally treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 1 cells (serum-free line) on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting tumour volumes as a function of time for 5 individual female mice treated with estradiol and tamoxifen (E2 + TAM). The shaded area represents the range of tumour volumes for 5 estradiol treated mice.

Note that estradiol and tamoxifen therapy stimulated tumour growth in female mice.

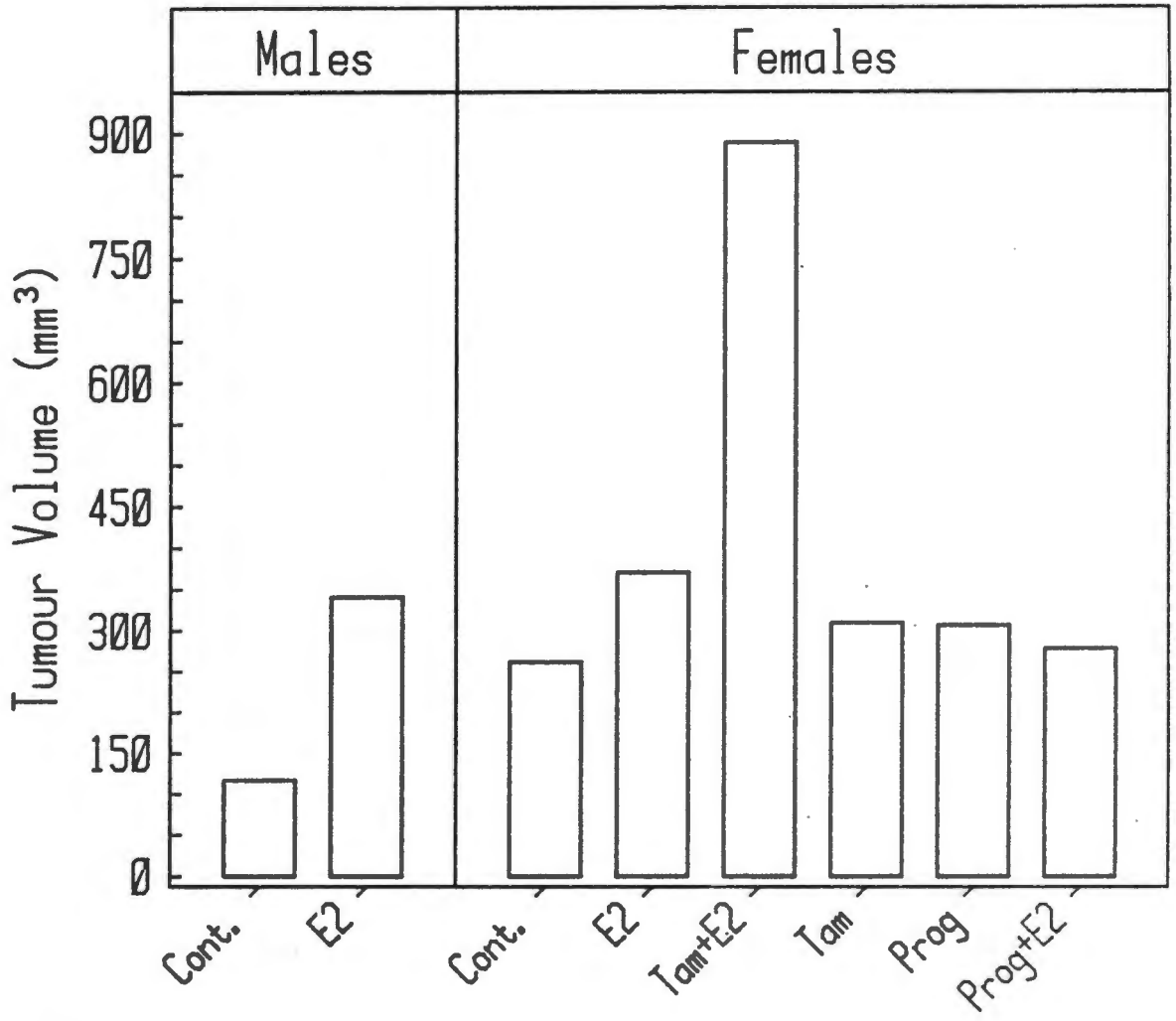


Figure 7d

The effect of hormonal therapy on the growth of UCT-Mel 1 tumours in nude mice

Male mice were implanted subcutaneously with estradiol (E2) pellets, whereas female mice were implanted subcunteously with estradiol (E2), tamoxifen (TAM), progesterone (PROG), TAM and E2 or PROG and E2 pellets. Twenty-four hours later 10^6 UCT-Mel 1 cells (serum-free line) were inoculated into these mice. Tumour volume was measured 28 days later. Note the following:-

(a) Estradiol stimulated the growth of tumours in male mice.

(b) Estradiol slightly increased the size of tumours in female mice.

(c) Tamoxifen and E2 together, stimulated tumour growth in female mice.

Experiment (x)

Table 3.11

Sex	Females							
Operation	Sx				Cx			
Treatment	Cnt	E 2	E 2 TAM	TAM	Cnt	E 2	E 2 TAM	TAM
Tumour No.	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
T(del)100	36	31	22	26	32	27	26	29
Td 200	10	10	8	6	10	7	6	9

Female mice were assigned to 8 treatment groups, 4 of which were sham-operated animals and the other oophorectomised animals (Table 3.11). Animals in each group then received no hormone therapy or they were treated with E2 or tamoxifen, alone or in combination. UCT-Mel 2 cells were injected at a dose of 10^6 cells.

These results are presented in Figs. 8a, b, c, d and e. In sham-operated animals E2 alone had no effect on tumour size (Fig. 8a) whereas tamoxifen increased tumour size (Fig. 8b). As can be noted from Figs. 8c,d and f in castrated animals E2 and tamoxifen were both slightly stimulatory for tumour growth. E2 and tamoxifen in combination resulted in a further increase in tumour size (Figs. 8e and 8f). In sham-operated animals the T(del)100 was reduced to 22 days from 36 days and in castrated animals to 26 from 32 days (Table 3.11). Fig. 8f depicts tumour size 41 days after inocuation of cells and the stimulatory effect of tamoxifen and E2 can readily be noted.

FIGURES 8a and 8b

Figure 8a

Growth of human melanomas in estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 2 cells (124') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 untreated control mice.

Note that estradiol therapy had no effect on the growth of tumours in female mice inoculated with UCT-Mel 2 cells.

Figure 8b

Growth of human melanomas in tamoxifen treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 2 cells (124') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual mice treated with tamoxifen. The shaded area represents the range of tumour volumes for 5 untreated control mice.

Note that tamoxifen increased slightly the tumour growth in female mice.

Figure 8a

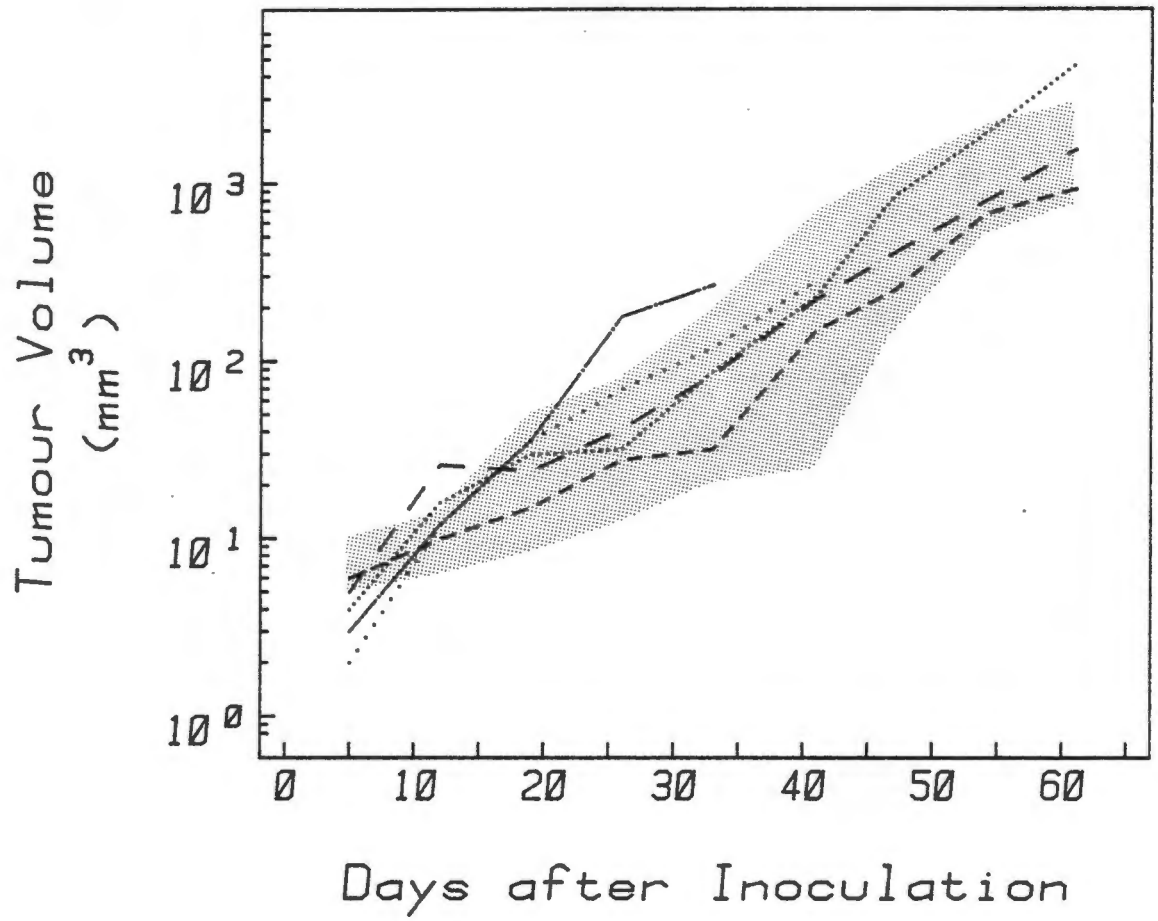
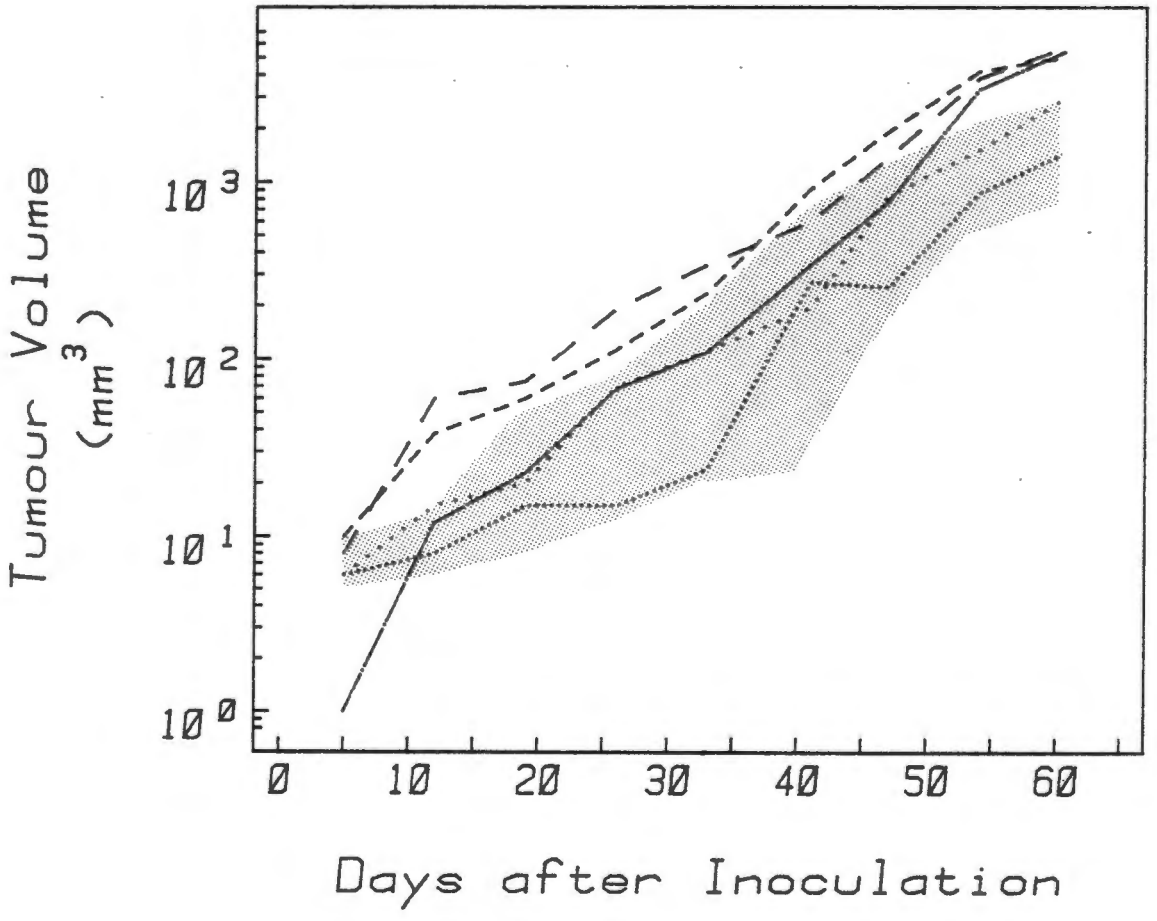


Figure 8b



FIGURES 8c and 8d

Figure 8c

Growth of human melanomas in ovariectomised and estradiol treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 2 cells (124') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual ovariectomised female mice treated with estradiol. The shaded area represents the range of tumour volumes for 5 ovariectomised untreated mice.

Note that estradiol treatment had a slightly stimulatory effect on the growth of the tumours in ovariectomised female mice.

Figure 8d

Growth of human melanomas in ovariectomised and tamoxifen treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 2 cells (124') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual ovariectomised female mice treated with tamoxifen. The shaded area represents the range of tumour volumes for 5 ovariectomised control mice.

Note that tamoxifen increased slightly the growth of the tumours in ovariectomised female mice. This was also found in the case of sham-operated female mice (see Fig. 8b).

Figure 8c

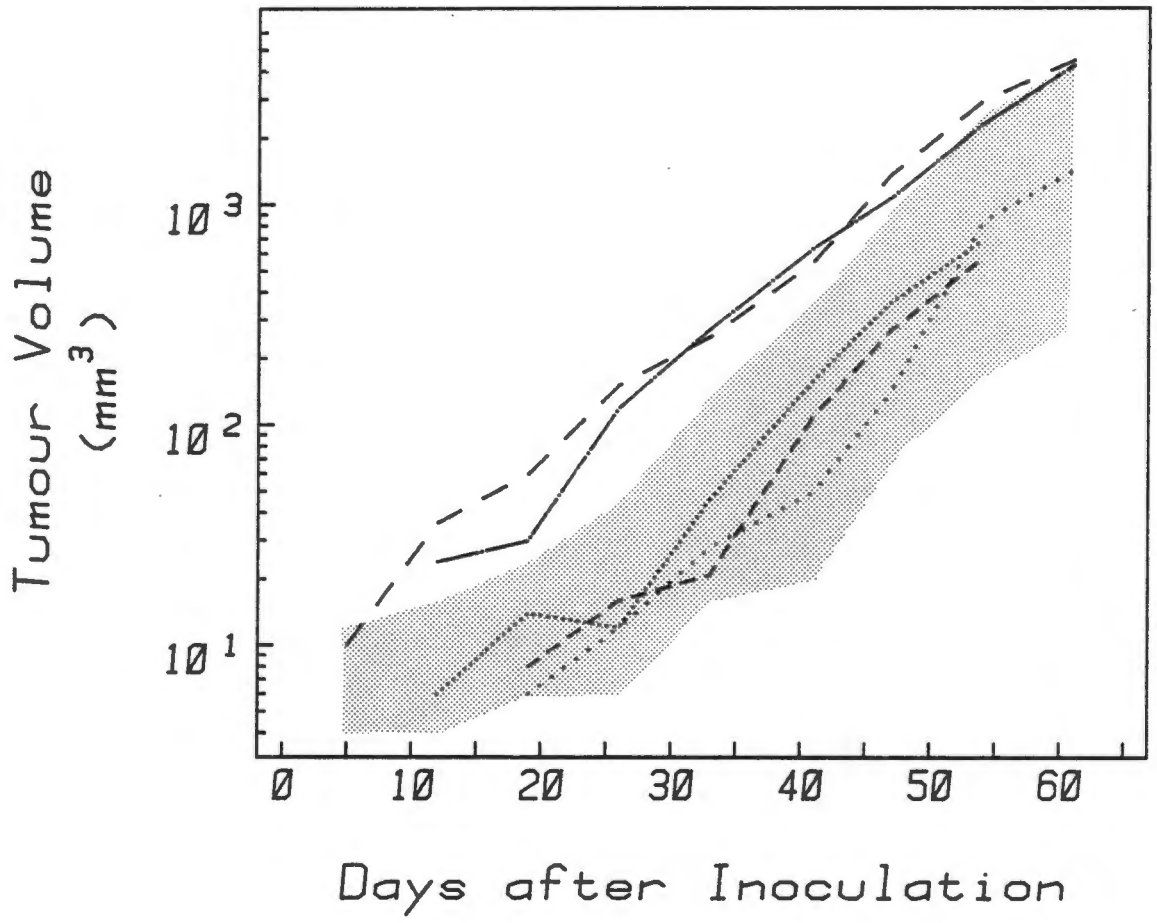
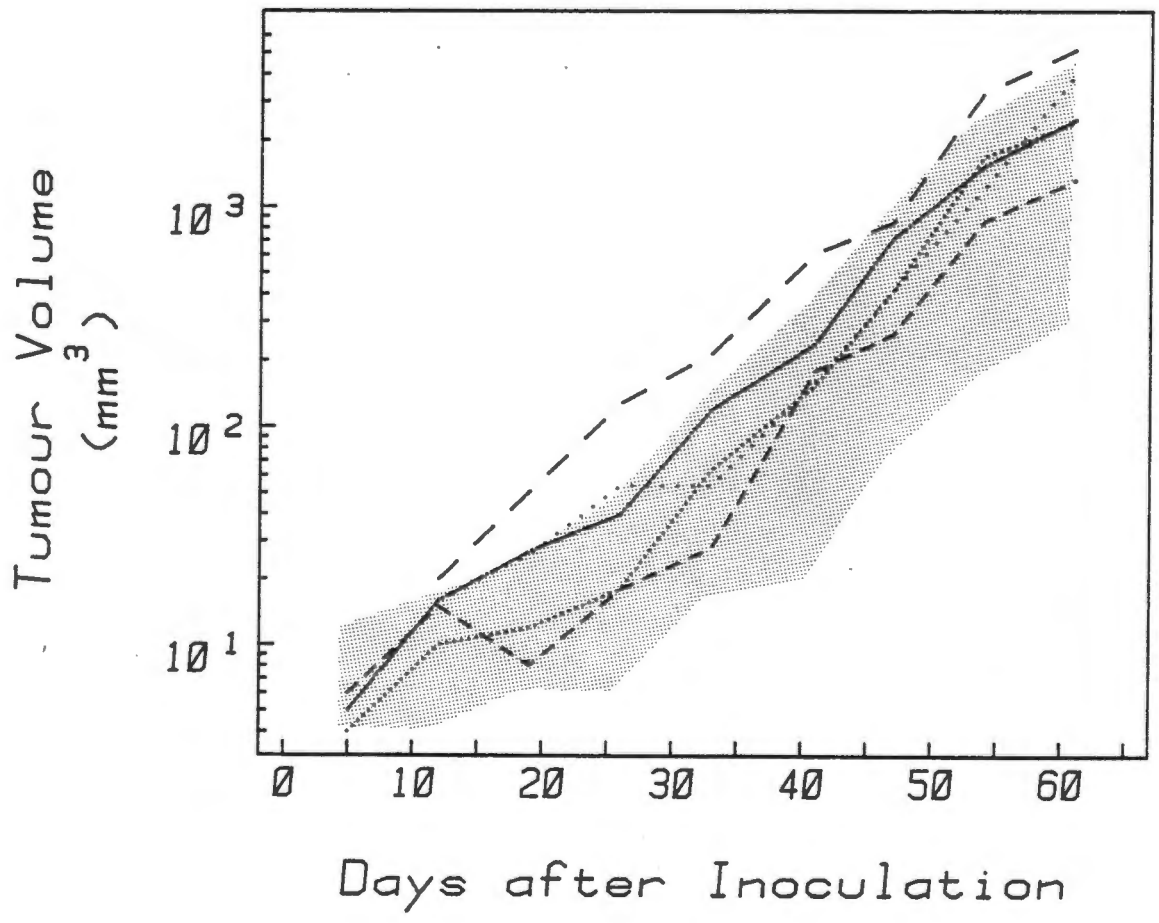


Figure 8d



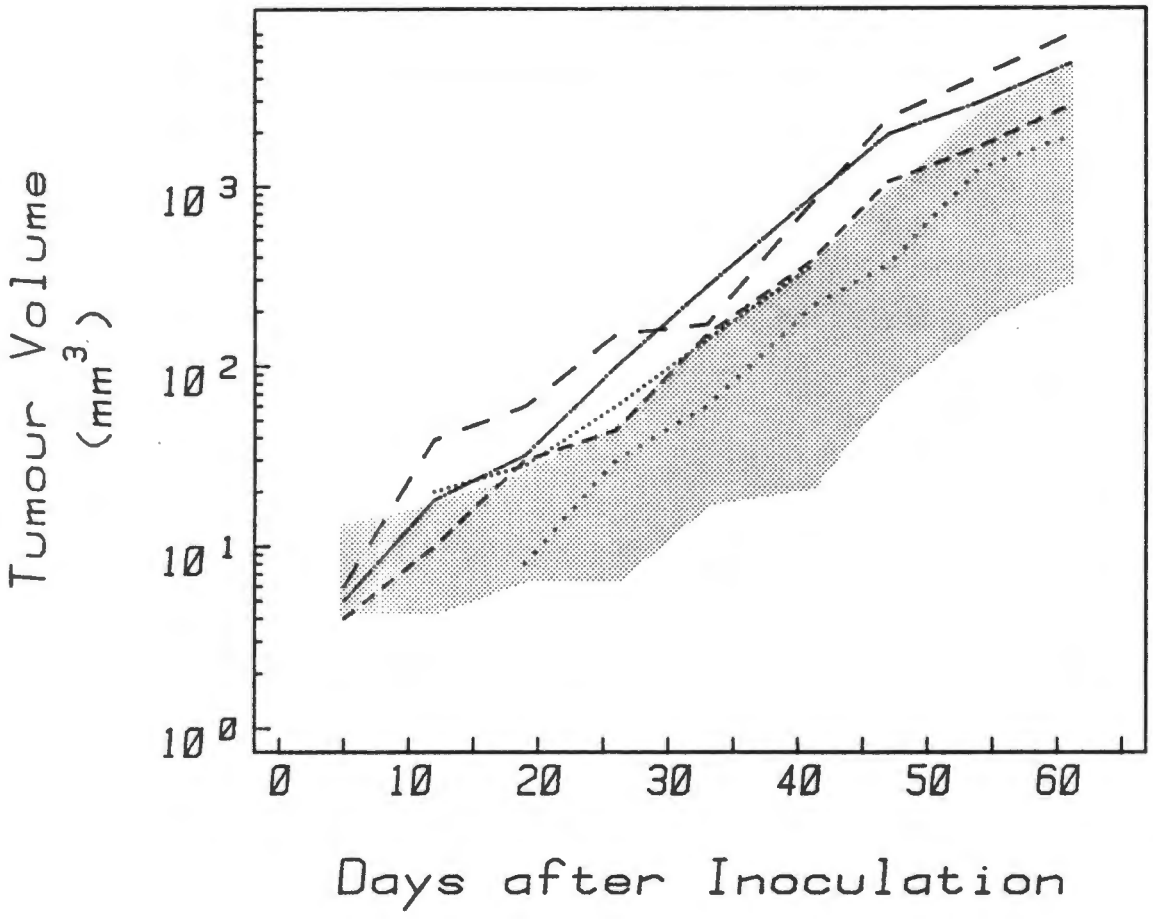


Figure 8e

Growth of human melanomas in ovariectomised and hormonally treated female nude mice

Female mice were inoculated subcutaneously with 10^6 UCT-Mel 2 cells (124') on day 0 and tumour volumes determined at weekly time intervals. The dashed lines in the figure were constructed by plotting the tumour volumes as a function of time for 5 individual ovariectomised female mice treated with estradiol and tamoxifen (E2 + TAM). The shaded area represents the range of tumour volumes for 5 ovariectomised control mice.

Note that estradiol and tamoxifen therapy stimulated the growth of tumours in ovariectomised females.

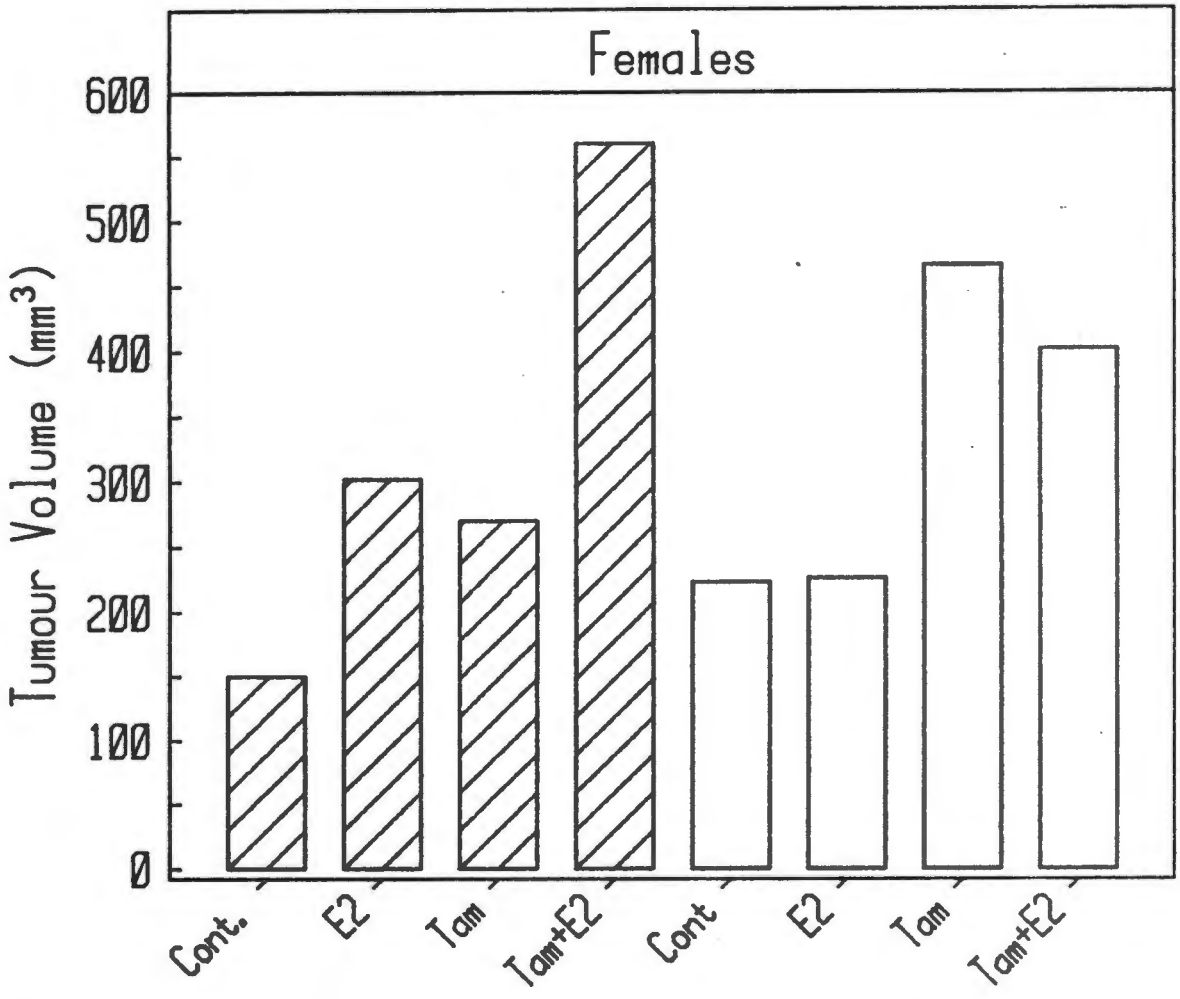


Figure 8f

The effect of ovariectomy and hormonal therapy on the growth of UCT-Mel 2 tumours in female nude mice

Female mice were castrated and 3-4 weeks later estradiol (E2), tamoxifen (TAM) or TAM and E2 (TAM + E2) pellets were implanted subcutaneously. Twenty-four hours later 10^6 UCT-Mel 2 cells (124') were inoculated into ovariectomised and sham-operated animals. Tumour volume was measured 41 days later. Note the following:-

(a) Ovariectomy alone reduced the tumour size in female mice.

(b) Tamoxifen and E2 enhanced profoundly the growth of tumours in ovariectomised and sham-operated females.

▨ - castrated; □ - sham-operated

Tumour dormancy and endocrine control.

The fact that the tumorigenicity of certain of the cell lines could be influenced by hormonal manipulation suggested a means of studying tumour dormancy in as much as this can be equated with the survival of tumour cells that do not proliferate to form a tumour yet retain the potential to do so when triggered by the appropriate stimulus.

Two combinations of cell lines and animal recipients were chosen to perform these experiments.

In the first, 1.5×10^5 UCT-Mel 2 cells were inoculated into 20 castrated male mice on day 0. The mice were divided into four groups, as shown in Fig. 9a. Group 1 received no replacement therapy and, as expected no tumours developed over the following year. Mice in the second group received DHT pellets on day -1 and again on day 21 (i.e. after an effective pellet dose-time had expired) and in this group tumours developed in 4/5 animals. The third group received DHT pellets on day 6 and day 27; 2/5 mice in this group developed tumours. The fourth group received a pellet on day 27 and 1/5 mice in this group developed a tumour.

In the second experiment ovariectomised female mice were inoculated with 2×10^5 UCT-Mel 3 cells on day 0 and E2 supplementation was given to the different groups on day -1, day 6, day 21, day 27 and day 34 (Fig. 9b). Here, once again as expected, mice that received no E2 treatment did not develop tumours during the course of the ensuing 7 months. Tumours were observed in all of the groups that received E2 pellets - even when these were administered as late as 34 days after the inoculation of tumour cells into an hormonally unfavourable environment.

The time that I waited for tumours to develop before scoring the mice as negative was certainly adequate since when I reviewed the total experience I had had with inoculation of human melanoma cells into nude mice (i.e 1065

CELL LINE - UCT-Mel 2

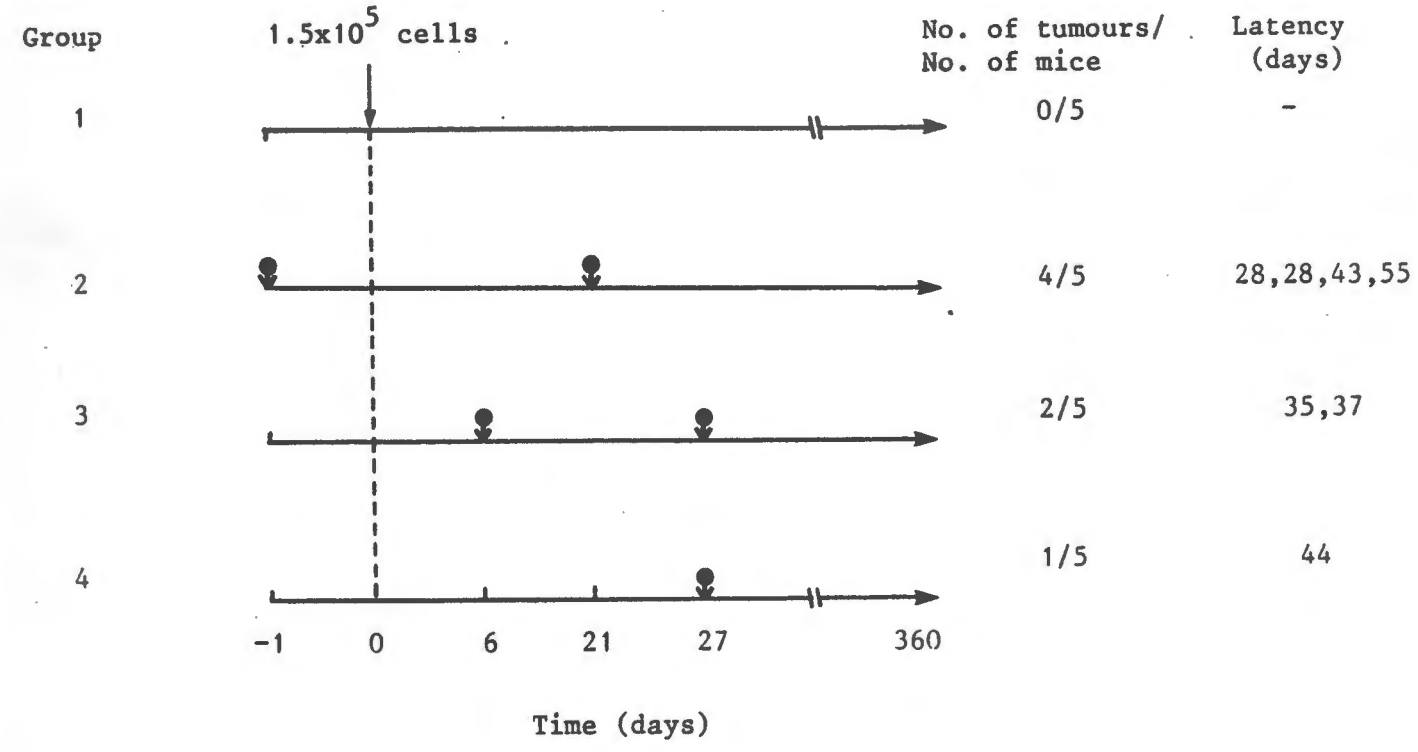


Figure 9a

Effect of DHT on the growth of UCT-Mel 2 in castrated nude mice

Male mice were castrated and 3-4 weeks later 1.5×10^5 UCT-Mel 2 cells (*in vitro* passage number 124') were inoculated subcutaneously into these animals on day 0. The mice were divided into four groups:-

Group 1 received no DHT pellets;

Group 2 received DHT pellets on day -1 and day 21 (the pellets were effective for a period of 21 days);

Group 3 received DHT pellets on day 6 and 27 ;

Group 4 received DHT pellets on day 27.

Note that castration alone inhibited the tumour growth in male mice; DHT therapy restored the tumour growth in 4 out of 5 mice in group 2, in 2/5 mice in group 3 and in 1 out of 5 mice in group 4.

CELL LINE - UCT-Mel 3

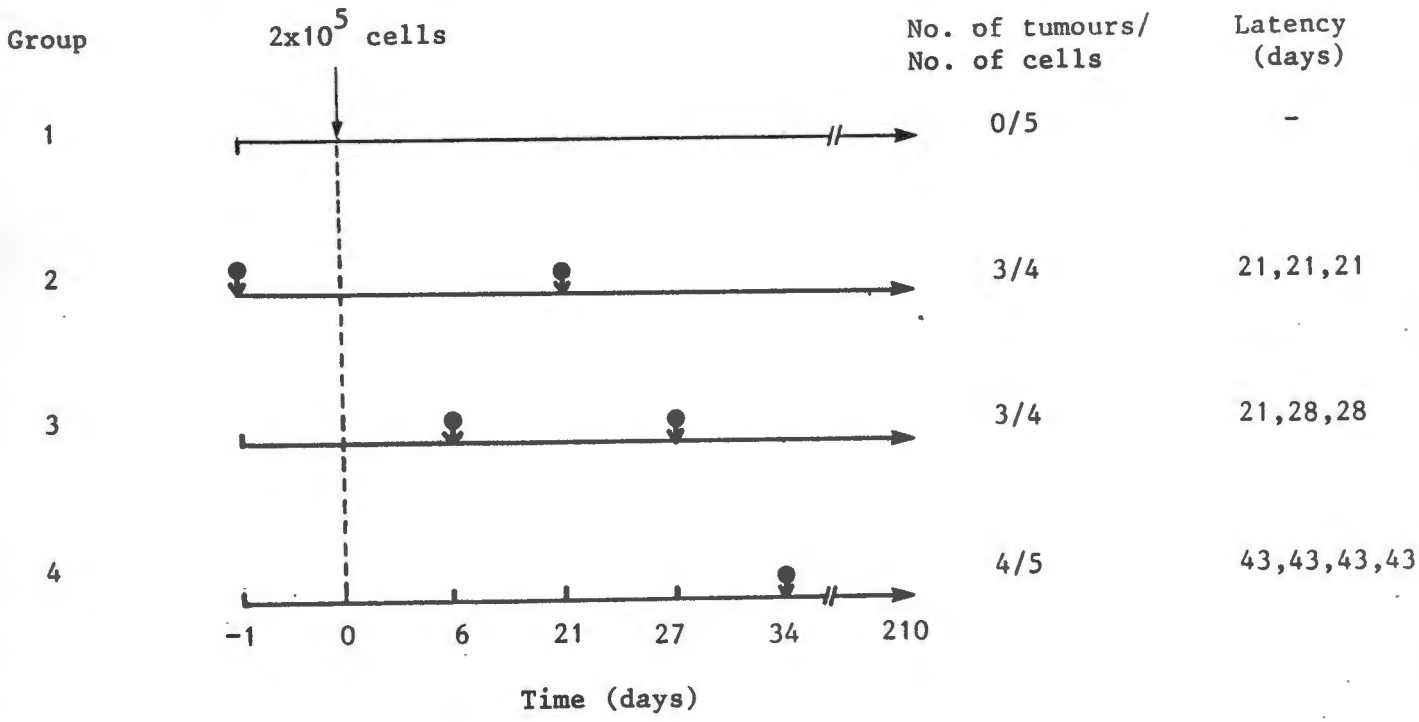


Figure 9b

Effect of estradiol on the growth of UCT-Mel 3 in ovariectomised nude mice

Female mice were ovariectomised and 3-4 weeks later $2x10^5$ UCT-Mel 3 cells (*in vitro* passage number 69') were inoculated subcutaneously into these animals on day 0. Mice were divided into four groups:-

Group 1 received no E2 pellets;

Group 2 received E2 pellets on day -1 and day 21 (the pellets were effective for a period of 21 days);

Group 3 received E2 pellets on day 6 and 27;

Group 4 received E2 pellets on day 34.

Note that mice that received no E2 treatment did not develop tumours during the course of the ensuing 7 months. Tumour growth was observed in the groups of mice that received E2 therapy even when this was administered as late as 34 days after the tumour cells were inoculated.

animals) 81% of them developed tumours. A plot of cumulative tumour appearance as a function of time after inoculation of the cells showed that 95% of the animals that were going to develop tumours did so in 6 weeks of inoculation and all of them within 85 days of inoculation (Fig. 10).

DISCUSSION

The results that I report in this chapter support the view that the growth of human melanoma cell xenografts in the nude mouse is an endocrinologically controlled phenomenon. The following experiments attest to this fact:-

(i) Inocula of 10^5 UCT-Mel 2 cells were tumorigenic in male mice; marginally tumorigenic in female mice and non-tumorigenic in castrated males (Experiment (i)). When females were oophorectomised the take rate was not affected, suggesting that the effects of orchidectomy in males are not secondary to the hypothalamic consequences of castration but rather to androgenic deprivation.

The preference shown by limiting numbers of UCT-Mel 2 cells for an androgenic environment were not consistently observed. In Experiment (iii) and Experiment (iv), for example, larger inocula did not cause tumours in 100% of sham-operated male mice and equivalent inocula (Experiment (iv)) formed tumours in castrated males. I have no good explanation for this variable behaviour. The experiments were done at different times so it might well be that environmental factors such as feed, time of year, light-dark cycling, temperature or any of the many other variables that are known to affect the performance of laboratory animals in complex experiments (Murthy and Russfield, 1970; Hoffman, 1973; Kledzik and Meites, 1974) may have caused the variability. Alternatively, subtle technical factors such as the density of cells prior to harvesting or the preparation of cells for inoculation or the inoculation procedures could have been responsible. Variability could

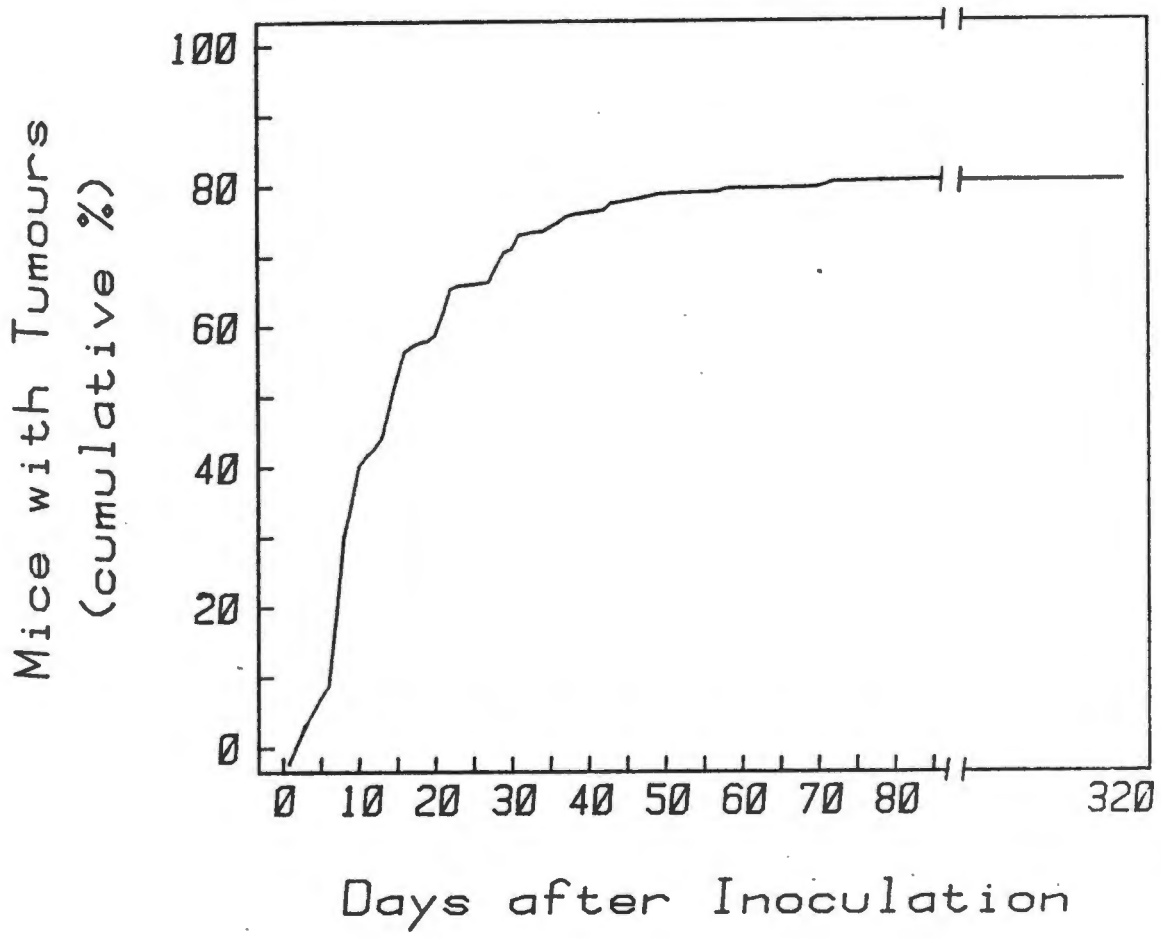


Figure 10

Appearance of tumours as a function of time after tumour cell inoculation

One thousand and sixty five mice were inoculated subcutaneously with varying numbers of tumour cells from UCT-Mel 1, 2, 3, 4, 5 and 7 cell lines. Eight hundred and sixty four mice developed tumours. The graph represents overall tumour development in the mice as a function of time.

Note that 95% of mice that were going to develop tumours did so within 42 days of inoculation and all of them within 85 days of inoculation of tumour cells.

also have been due to the fact that the strain of mice used was not inbred and genetic variation would therefore be expected among littermates.

Whatever the cause for the variability may have been, it is important to recognize its occurrence to stress the need for experimental systems involving nude mice that are stable and reproducible over a prolonged period of time.

(ii) Experiments showing an effect of E2 on melanoma cell growth were more conclusive. Tumour growth following inoculation of UCT-Mel 1, 2, 3 and 7 was stimulated by E2. E2, in fact, stimulated growth in all 4 of the melanoma cell lines investigated. In experiment (vii) (Fig. 5a) E2 stimulated the growth of UCT-mel 1 in females and not in males and in experiment (v) (Figs. 3a,b,c,and d) E2 stimulated the growth of UCT-Mel 3 tumours in both normal and castrated male and female mice. The effects of this hormone were however, far more pronounced in castrated animals (Figs. 3c, d and e). The effects of E2 on the growth rate of UCT-Mel 7 were also more pronounced following castration in male mice (Figs. 4d and g). Thus the endocrinological status of the host animal was an important factor in determining the manner in which it responded to hormonal manipulation.

It is of interest to note that one of the experiments in which the E2 effect was most remarkable was that in which E2 implantation, even as late as 34 days post-inoculation of tumour cells, rescued a non-tumorigenic inoculum of 2×10^5 UCT-Mel 3 cells in female mice (Fig. 9).

(iii) DHT was of interest as it had both positive and negative effects on tumour growth.

Both experiments (iii) and (iv) show that DHT promotes tumour growth by UCT-Mel 2 cells though not as much as E2. Experiment (iii) shows that DHT was especially effective in promoting tumour development in castrated female mice. DHT also stimulated tumour growth by UCT-Mel 3 cells especially in female mice - both control and castrated (Experiment (v) - Figs.3e, f and g). Experiment (vi) shows that tumour growth of UCT-Mel 7 was markedly increased by this

androgen in castrated male mice (Fig. 4f and g). However, in the case of UCT-Mel 1 cells, DHT exerted an inhibitory influence on tumour growth. Experiment (viii) and Figs. 5c, d and e show that in both normal and castrated male mice the androgen diminished the tumour growth rate.

The effects of androgens and estrogens on melanoma growth in vivo is therefore complex and is probably dependent on a number of factors of which the endocrinological status of the host is one of the more important. The particular cell line investigated is obviously also important because my data show that variation in sensitivity to both androgens and estrogens occurs among the different melanoma cell lines used.

Another important lesson has emerged from my studies. If one wishes to show an effect of a modulating compound on tumour growth in the nude mouse, one has the best chance of doing so if the tumour cell dose is first titrated accurately to determine the tumorigenic threshold. It is clearly in the neighbourhood of this threshold that the system would be most sensitive to the effects of hormones or other regulatory compounds. The likelihood of observing a stimulatory effect would be greatest when using an inoculum size that was just sub-tumorigenic. Conversely, the chances of showing an inhibitory effect would be optimised if an inoculum size was used that was reproducibly but just sufficient to cause tumors in all animals tested.

This leads me to the next point concerning the development of a reliable assay system for studying growth modulation of xenografted human tumours in the nude mouse: of all of the measurements that can be made to quantitate the effects of regulatory compounds, tumorigenesis is the most robust, the delay time less so and the doubling time or growth rate least of all. It is apparent, in fact, from simple observation of the graphs that I present, that when E2 had an effect this was not only upon the growth rate of the tumour. It was also upon tumorigenicity (Figs. 2a;2b;3d;7a and Tables 3.5; 3.6; 3.10) or upon the delay time (Figs. 2b; 2c;2d;3c;3d;5a and Tables 3.5; 3.6; 3.8).

I would thus suggest that compounds are assayed for their regulatory activity by scoring for tumorigenicity of marginally tumorigenic or sub-tumorigenic inocula. It may also be possible, with well-devised protocols, to score for metastasis - another nominal quantity. I would not recommend that Gompertzian parameters be relied upon for the purpose of quantitating tumour growth in this experimental context.

I am not able to provide a definitive mechanism for the mode of action of E2 in the experiments in which I showed an effect. I am, however, able to offer the following comments and speculations.

Firstly, E2 implantation enhanced the tumour take rate and thus, presumably, the propitiousness of the host environment for xenograft acceptance, survival and proliferation. This result is not consistent with the oft-repeated observation (White, 1959; Nathanson et al., 1967a; Cochran, 1969; Shaw et al., 1978; 1980) that the prognosis of human malignant melanoma is better in females than in males or with the observation that, in one very clear cut experiment at least, (Experiment (i)) a marginally tumorigenic dose of UCT-Mel 2 cells produced tumours in 5/5 male mice but in 2/5 female mice.

It is noteworthy that the anti-estrogen, tamoxifen, not only failed to inhibit the stimulatory effects of E2 but, on 3 occasions, appeared to synergise with E2 in promoting growth (Experiments (viii), (ix) and (x); Figs. 6c,6d; 7c,7d; 8e,8f).

A similar paradoxical effect of tamoxifen on the growth of a human estrogen-responsive endometrial carcinoma in the nude mouse was observed by Satyaswaroop et al.,(1984) and it is well known that tamoxifen may be fully estrogenic in the mouse with respect to certain tissues (Harper and Walpole, 1967; Jordan et al., 1978; Terenius, 1971) whereas it is usually anti-estrogenic in humans. Furthermore, Gallez et al.,(1973) showed that another human anti-estrogen (nafoxidine) prevented the regression of rat mammary tumours after ovariectomy. Had E2 acted directly on the human melanoma cells

I would then thus have expected tamoxifen to have inhibited tumour growth. The fact that it did the opposite suggests that both its effect and the effects of E2 were indirect and mediated by an action on murine tissues.

My experience thus suggests that the actions of E2 were complex and did not simply involve the stimulation of melanoma cell proliferation by the conventional sequence of biological events in which E2 bound to a specific receptor protein for transfer into the nucleus where a stimulatory interaction between genome and receptor-hormone complex occurred (McGuire et al., 1975). My failure to detect estrogen receptor activity in any of the melanoma cell lines that I studied is in accordance with this suggestion and with the observations of Fisher et al., 1978.

The above observations are consistent with the view that estrogens did not act directly upon the melanoma cells but rather that their effects were indirect in the sense:-

(i) that they were mediated by an action on the pituitary or hypothalamus which leads to the induction of growth factor release or the suppression of inhibitory factor release.

(ii) that they involved the release of "paracrine" estromedins from neighbouring cells that stimulated tumour cell proliferation (Sirbasku and Benson, 1979) or

(iii) that their action was entirely upon supporting systems for tumour development such as angiogenesis (Folkman, 1974), basement membrane synthesis (Kramer et al., 1980),fibronectin synthesis (Kramer et al., 1980) etc.

Fortunately these alternatives are all amenable to experimental enquiry and it is my intention to pursue these studies with the benefit of the experience I have had to date.

CHAPTER 4

CHAPTER 4

THE METASTATIC SPREAD OF HUMAN MELANOMAS IN THE NUDE MOUSE.

Of all the clinical features that characterize malignant neoplasms, it is their capacity for local invasion and widespread dissemination that distinguish them most cogently from normal cells or from their benign counterparts. Most deaths from this group of diseases and much of the disability that they cause are attributable to metastatic spread and there is no doubt that hopelessness, in the individual case, is directly related to dissemination.

It is scarcely surprising, therefore, that so much research effort has been devoted to the study of metastasis and that such a voluminous literature has grown around this particular complication of malignant disease.

It is apparent, from a survey of this literature, that most of the research that has been done into metastasis has taken the view that the spread of malignant tumours is the end result of a number of processes operating sequentially. When considered against the background of biological knowledge generally or in the light of information that has been gleaned from descriptive observations, these processes are seen to have certain necessary requirements for their successful completion. Experiments are then designed to explore the need for the particular requirements or, frequently, they assume that the requirement exists and the research is directed at defining the way in which it is met.

These processes may be summarised as follows:-

(i) Cells dislodge from the primary tumour

This process is seen to require circumstances such that forces promoting separation of the cells (lytic enzymes, tissue movement or handling of the tumour) exceed those that are responsible for cohesion or adherence (cell junctions, cell association molecules and mechanical restraints). Experiments have been performed to show that malignant or transformed cells may, indeed, produce proteolytic enzymes with collagenolytic or tryptic specificity (Liotta

et al., 1981; Ossowski et al., 1973; Strauch, 1972) the tumour cells adhere to each other and to a substrate less tenaciously than do normal cells (Coman, 1961; Willis, 1973), or that structures such as gap junctions or molecules that are involved in cell-cell adhesion are deficient in malignant cells (Poste, 1977; Weinstein et al., 1976; Sheridam, 1976; Weiss, 1976); and that surgical manipulation of tumours causes the release of showers of tumour cells into the venous circulation (Golinger et al., 1977; Griffiths et al., 1973; Salsbury, 1975).

(ii) **Cells penetrate the microbarriers that separate the primary tumour from the adjacent tissues or from the lymphatic or vascular compartments.**

This penetration may precede dislodgement so that detachment of the tumour fragment is effected, mechanically, by the circulation or it may follow the detachment of motile cells. In any event cellular penetration requires the traversal of basement membranes and other structures. This, in turn, requires recognition structures on the tumour cells whereby they attach to the basement membrane or other tissue components and hydrolytic enzymes such as collagenase or plasminogen activator for the dissolution of the membrane and, finally, the capacity for locomotion. Correlations between laminin receptor density (Liotta, 1986; Hayman et al.,1981;Chung et al.,1979; Rao et al.,1983; Terranova et al.,1980; 1983), protease sectretion (Sylven and Bois-Svensson, 1960; Koono et al., 1974; Strauch, 1972), and migration capacity (Easty and Easty, 1974; 1984; Enterline and Coman, 1950; Burk, 1973) on the one hand and cellular metastatic potential on the other have been reported although these results have not always been consistent (Kinsey, 1960; Fidler, 1982; Fidler and Kripke, 1977; Hart and Fidler, 1981; Poste and Fidler, 1980; Talmadge et al., 1982).

(iii) **Cells survive in the blood stream or lymphatic vessels until their arrest in the first capillary filtration bed that they encounter.**

Cellular survival requires the presence of tumour-cell surface receptors for growth factors or nutrients or the lack of dependence on such factors. There is also a requirement for mechanisms for evading immune attack and circulating cells of the immune system and, finally, tumour cells should be sufficiently hardy in the mechanical sense, to withstand the buffeting environment of the blood stream.

Capillary arrest requires favourable interactions between the tumour cell surface and the capillary endothelial wall so enabling the circulating tumour cells to escape from the blood stream and attach to the vessel. Several reports (Chew et al., 1976) have implicated thrombogenesis in capillary embolisation by tumours and it has been suggested (Buck, 1973; Lunscken and Strauli, 1975) that denudation of the endothelial layer may promote adhesion by exposing collagenous or other binding sites or by removing the negative charge on the endothelial cell that would presumably constitute an electrostatic barrier attachment of negatively charged tumour cells (Ozakit et al., 1971). Cell surface negative charge may also be reduced by defective sialyl transferase activity (Robbins and Nicolson, 1975; Roblin et al., 1975) that diminishes terminal sialic acid residue density on membrane glycoproteins.

Other cell surface changes have been associated with neoplastic transformation (e.g. susceptibility to agglutination by plant lectins (Rapin and Burger, 1974; Nicolson, 1974)) and generally speaking, the implication is made that such changes somehow facilitate cell-endothelial interaction that favour capillary arrest.

(iv) **Cells that come to rest in the capillary bed penetrate the vessel wall. They then infiltrate the adjacent tissues and proliferate.**

These processes would require lytic and migratory capabilities such as those already mentions in (iii) and (iv) above. Furthermore, new environments in which they find themselves should provide an auspicious milieu for the growth of the micrometastasis in both the immunological and endocrine sense.

(v) **The metastatic deposit acquires a substantive stroma and blood supply. A major restraint upon growth is thus removed. The metastasis is now able to function as a source of tertiary metastatic deposits.**

These processes require favourable host-tissue interactions as exemplified by the response of host cells to tumour-cell products such as angiogenic factor (Folkman, 1974a,b; 1975; Folkman et al., 1971; Folkman and Tyler, 1977) or factors that promote infiltration of metastasis. Mesenchymal cells provide a framework for cell survival and a possible source of paracrine controlling growth factor activity (Sporn and Roberts, 1985; Sporn and Todaro, 1980).

In addition to these experiments designed to examine a particular idea or aspect of the metastatic process, there are a number of observtaions of a more general nature that have come from the study of human neoplasms with experimental tumours.

In the first place, it is now clear that the mere presence of viable tumour cells in the circulation does not necessarily signify metastasis. Only a small fraction of such cells implant successfully (Salsbury, 1975; Fidler, 1970; 1975; Griffiths and Salsbury, 1965; Clifton and Agostino, 1962). From this we may infer that the probability that a cell that is metastatically equipotential with all other cells will arrest in the right place at the right time is small. Alternatively, only a small fraction of the cells that discharge from a primary tumour are stem cells that are capable of establishing a metastatic colony. Other cells would be terminally differentiated or transit cells with the capacity, perhaps, for a few divisions after which they would

die.

Secondly, it is well known - once again from both clinical and experimental observations - that metastases may manifest long after the primary lesion has been successfully treated and apparently a tumour-free period has elapsed. This phenomenon of quiescence followed by the reappearance of overt tumour at a distal site is referred to as "tumour dormancy". Several papers (Eccles and Alexander, 1975; Wheelock et al., 1977) have appeared on this most interesting aspect of metastatic spread since the phenomenon was first validated experimentally by Fisher and Fisher in 1959.

Thirdly, studies that have measured the fate and distribution of tumour cells introduced directly into the circulation have shown conclusively that, for certain kinds of cells, their distribution is not random. One finds, rather, that an "organotropic" predeliction exists for the cells to arrest, reproducibly, in certain specific organs. Fidler's experiments (1978) have been most convincing in that he has isolated B16 mouse melanoma sublines that metastasise preferentially to particular organs or tissues. Clinical and autopsy studies in humans have similarly shown that tumours belonging to a particular class (e.g. breast) have a significant proclivity for spread to a particular set of organs (e.g. ovaries). In some cases this may be due to cell surface structures that form specific interactions between the tumour cell and complementary molecules on the capillary vascular endothelium of the preferred organ; in other cases the organ may offer a hormonal or nutritional milieu that favours growth of the cell type concerned.

Many ingenious <u>in vitro</u> and <u>in vivo</u> experimental systems have been devised to study invasion and metastasis and the results that have been obtained with their use have enabled one to identify certain characteristics that can reasonably be associated with metastatic cells and the tissues that they invade. These models have recently been the subject of informative and comprehensive reviews (Easty and Easty, 1984; Poste, 1982; Carr and Carr,

1982; Gershman, 1982).

Generally speaking, *in vitro* studies have used an experimental approach in which malignant cells were incubated with substrates for invasion that have varied from relatively simple macromolecular layers such as collagen gels (Schor, 1980; Yang et al., 1981) through composite organotypic cultures comprising structural proteins, mesenchymal and epithelial elements (Wolf, 1963; De Ridder et al., 1977), natural membranes (Easty and Easty, 1984; Hart and Fidler, 1978; Poste et al., 1980) or sponge matrices (Leighton, 1951); up to complex organ cultures (Wiseman and Steinberg, 1973; Weston and Abercrombi, 1967). These *in vitro* models, although artificial, have had the great advantages that they are relatively simple to construct and that they allow quantitative studies to be undertaken by, for example, the use of radioactive substrates or the counting of cells that have penetrated the assay matrix. Furthermore, *in vitro* systems have made it possible to test directly the processes that the various theories of invasion and metastasis predict. If, for example, one proposed that metastatic organotropism involved interactions between complementary recognition structures on tumour cells and the cells of the preferred organ, *in vitro* aggregation or rosette studies could be used to support this notion. Liotta (1986) has used this approach to elegant advantage to support his views that interactions with laminin and the secretion of type IV collagenase are important components of the invasive phenotype.

Although these simplified approaches have enabled investigators to take the metastatic cascade one step at a time, the light that they have shed has been circumstantial rather than definitive and the need for *in vivo* confirmation has usually remained.

The *in vivo* models that have been used have generally involved the study of tumour cell behaviour following grafting into the immunologically bland environment of a syngeneic or immunoincompetant host. Although many of the

studies in this category have been largely descriptive, the emergence of experiments of such workers as Fidler (1970; 1973b; 1974a; 1974b; 1976; 1978; 1982; Fidler and Nicolson, 1976; Fidler and Kripke, 1977; Fidler et al., 1984; Fidler and Hart, 1982) Nicholson (Nicolson et al., 1976; Nicolson and Winkelhake, 1975; 1976) Folkman (Folkman and Tyler, 1977; Folkman et al., 1971; Folkman, 1975; 1974a, b; 1971), Sordat (Sordat and Merenda, 1977; Sordat et al., 1978; Sordat and Bogenmann, 1980), Weiss (Weiss et al., 1974; Weiss, 1976; Weiss et al., 1983) and the Fishers (Fisher et al., 1969; 1970; 1983) have contributed materially to conceptual developments in metastasis research. The published proceedings of two recent symposia on this subject (Houchens and Ovejera, 1978; Sordat,(1982) and the excellent review by Fidler (Fidler et al., 1978) provide comprehensive accounts of the knowledge that has been gleaned from the study of animal tumours.

My concern has been largely with the development of procedures for the experimental study of the spread of human tumours and, particularly, using the nude mouse as a model for investigating the spread of human melanoma. Ossowski and Reich (1980) have used the embryonated hen's egg as a remarkably effective system for studying the growth and spread of a human epithelial tumour, and they were able, with this model, to demonstrate the importance of plasminogen activation in the metastatic process. The athymic mouse should provide an environment for xenograft acceptance that is every bit as accommodating as the chorio-allantoic membrane of the hen's egg. It should, moreover, have the theoretical advantages of phylogenetic proximity and close anatomical similarity to man coupled with the practical advantages of a longer period of time over which observations can be made. In the case of the egg one is limited to approximately two weeks; the nude mouse allows slowly growing tumours to be observed for periods up to one year.

A survey of the literature indicates that the nude mouse has not been particularly useful for the investigation of metastasis of human tumours.

Most authors have noted that human tumours implanted subcutaneously in this species give rise to well encapsulated lesions that remain localized at the site of inoculation until the death of the animal (Helson et al., 1975; Shimosato et al., 1976; Kameya et al., 1976; Giovanella et al., 1978; Sharkey and Fogh, 1979; Kanzaki et al., 1979; McCormick et al., 1983).

In the experiments to be done in this chapter I show that the nude mouse provides an excellent and informative model for the investigation of human tumour spread provided survival of the animal is prolonged by excision of the primary tumour.

MATERIALS AND METHODS.

Mice and cell lines

The mice and the cell lines used for these experiments have been described in detail in Chapter 1 and Appendix.

Experimental protocol

Mice received subcutaneous inocula of melanoma cells as described in Chapter 1. The tumours that developed were excised before they reached a size which was lethal to the host or when they had been present for a time that was appropriate for each experiment.

The mice were anaesthetized with ether and the tumours were removed, together with an ellipse of overlying skin. The tumours were usually circumscribed and could readily be separated from the surrounding normal tissue by blunt dissection. The skin incision was then closed with clips which were normally removed on the 4th or 5th post-operative day. Haemostasis did not present a problem during removal except in the case of UCT-Mel 3 and wound healing was invariably rapid without infection.

The animals were examined frequently for up to one year during the post-operative period for evidence of metastasis; weight loss provided the most

reliable evidence to indicate that this had occurred.

Detailed autopsies were prepared, under sterile conditions, on all mice that died spontaneously or upon animals that were killed when dissemination was diagnosed at the end of the period of observation. To increase the accuracy of lung metastasis observation at the time of autopsy, lungs were insufflated with 15% solution of Indian Ink and washed with Fekete's solution, which is a bleach preservative, that facilitates the detection of very small lung implants (Wexter, 1966). At autopsy samples of tissue were taken into buffered formalin for histology or into sterile tissue culture medium for inoculation into fresh recipients or for return to *in vitro* culture. These techniques have been described in Chapter 1.

RESULTS

In the first series of experiments to be completed, mice received 10^6 or $5x10^6$ melanoma cells from the various cell lines and 120 of these animals developed tumours (UCT-Mel 6 was consistently non-tumorigenic). When the tumours had grown to a size where they were considered to be life threatening to the hosts or when small areas of necrosis were seen in the overlying skin, the primary tumour was excised. At the time of excision, most of the tumours were circumscribed and easily separated from the fascial planes or muscles of the back. While attachment to the overlying skin was common, the tumours were usually mobile relative to the deeper tissues with no obvious clinical signs of local invasion. This was borne out by the fact that local recurrence was a rare phenomenon that was seen in only 8 cases and then exclusively with UCT-Mel 2 and 3.

Mice bearing tumours derived from UCT-Mel 3 consistently showed a clotting disorder that I have not been able to elucidate in detail. I occasionally saw quite extensive haemorrhage into the tissues surrounding these tumours (Fig. 4.1) and, at the time of excision of the mass, it was

Figure 4.1

A mouse with extensive haemorrhage into the tissue which surrounded a UCT-Mel 3 tumour.

A male mouse was inoculated subcutaneously with 10^6 UCT-Mel 3 cells (*in vitro* passage number 69'). This tumour cell line frequently gave rise to haemorrhagic tumours.

The picture illustrates extensive subcutaneous haemorrhage which surrounded a UCT-Mel 3 tumour. The tumour had been removed through the visible incision prior to the photograph being taken.

usually only with some difficulty and the patient application of local compression that haemostasis could be secured. Blood samples taken from these mice formed loose clots that retracted poorly. The blood platelets appeared normal in appearance and number but that was as far as I was able to take the investigations of this interesting disorder. It certainly merits further study.

After the animals had the tumours excised they were monitored for periods of up to one year for signs of metastasis. Weight loss proved to be a very useful and reliable clinical indication of the fact that dissemination had taken place.

All of the tumorigenic lines, with the exception of UCT-Mel 7, gave rise to metastases with frequencies that ranged from 1/17 (UCT-Mel 1) to 35/35 (UCT-Mel 3). UCT-Mel 2, 4 and 5 disseminated with similar frequencies of approximately 10% (Table 4.1). Secondary deposits, in tests of the most aggressively metastatic line, UCT-Mel 3, were observed in the lungs, gonads, kidney, liver, intestines and abdominal cavity. In the other four cases the lung was the only site of secondary deposition.

Histological examination of the metastases showed appearances that were, for the most part, consistent with the diagnosis of haematogenous dissemination (Fig. 4.2), although in certain cases (e.g. Fig. 4.3) the metastases seemed to arise from spillage into serous cavities when they manifest as nodules that burrowed into the substance of the liver or the lung with a clear line of demarcation between normal tissue and tumour tissue and no sign of infiltration.

Selection for the metastatic phenotype

I hoped that, by repeated *in vivo* passaging of tumours derived from secondary deposits, I would be able to develop sublines that were more consistently metastatic. I thus transplanted, subcutaneously, fragments of metastatic tissue from tumours derived from each of the lines into replicate mice.

FIGURE 4.2

Figure 4.2

Metastases of human melanoma in the nude mouse.

(a) Metastasis of non-pigmented epithelioid cell type malignant melanoma (UCT-Mel 3) invading peripheral sinuses of a lymph node. The lymph node is represented at this stage by collections of small darkly staining lymphocytes.

(b) Metastasis of UCT-Mel 3 compressing a normal adrenal gland seen at top left. The tumour metastases are seen as nodules of undifferentiated cells (note mitoses) and the adrenal gland as columns of highly coloured cells with pale foaming cytoplasm.

(c) Two metastases of UCT-Mel 3 (top left and middle right) in kidney. The kidney shows glomeruli and proximal convoluted tubules while the more irregular shaped islands of tumour are composed of undifferentiated non-pigmented cells.

The scale marker in (a) represents 60 μm and in (b) and (c) 80 μm.

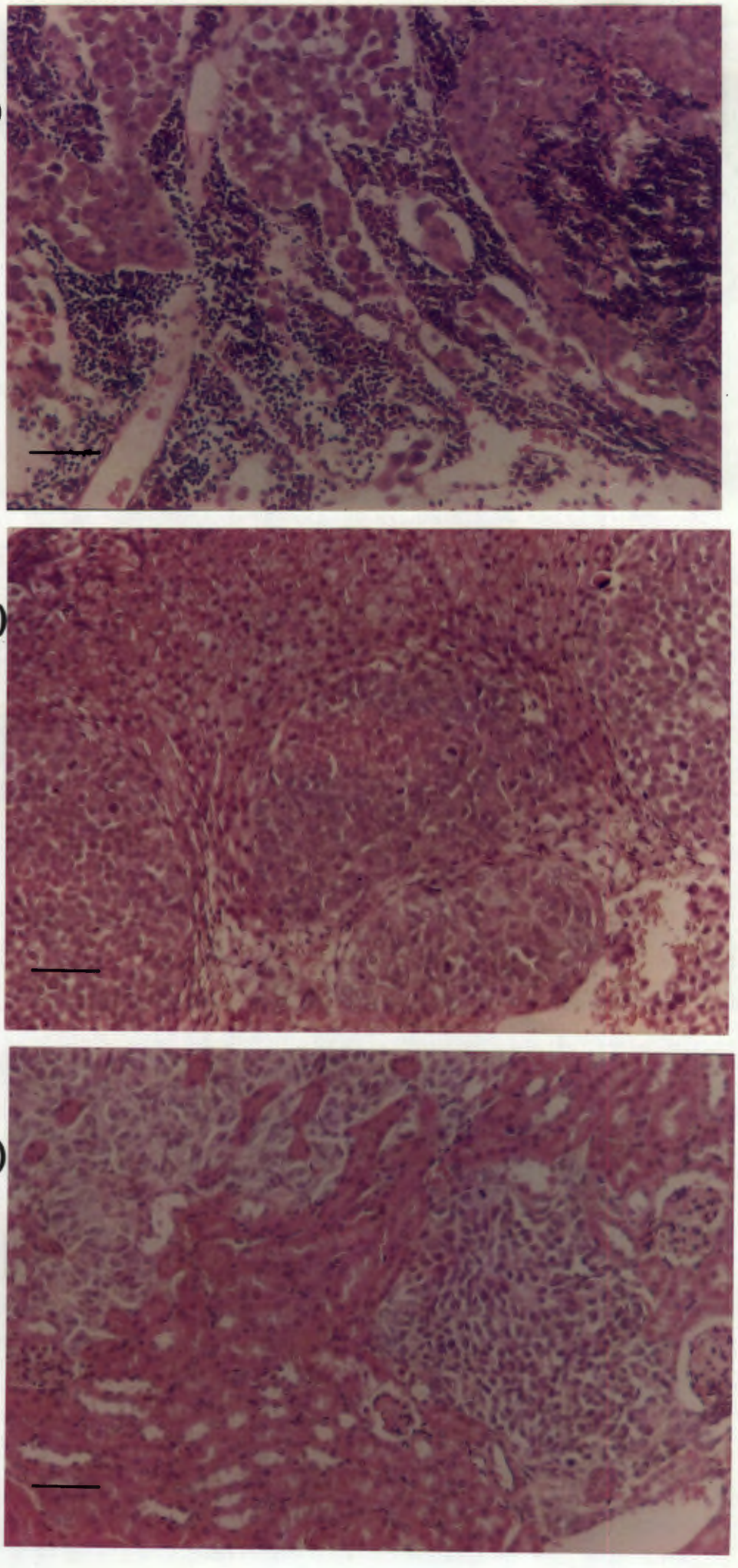


Figure 4.2

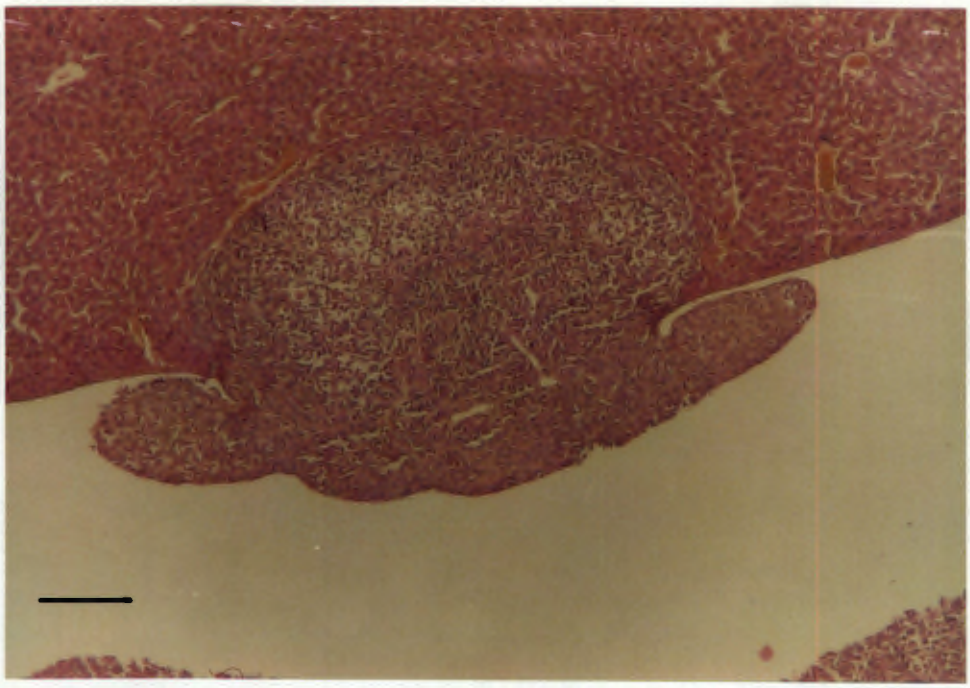


Figure 4.3

Metastasis of human melanoma in the nude mouse.

Metastasis of UCT-Mel 3 protruding into liver parenchyma from the capsular surface. Undifferentiated tumour cells can be seen forming a nodule on the peritoneal side and then pushing their way into the underlying normal liver.

The scale marker represents 200 μm.

TABLE 4.1

METASTASES IN NUDE MICE

CELL LINE	NUMBER OF MICE	METASTASES		MAXIMUM PERIOD OF OBSERVATION (days)	SITE OF METASTASES
		No.	%		
UCT-Mel 1	17	1	6	260	Lung
UCT-Mel 2	26	3	12	380	Lung
UCT-Mel 3	35	35	100	190	Lung Gonads Kidney Liver Intestine
UCT-Mel 4	10	1	10	270	Lung
UCT-Mel 5	22	3	14	313	Lung
UCT-Mel 7	10	0	0	370	-

When tumours formed locally they were excised and passaged, as subcutaneous fragments, and the recipients from whom the tumours were removed were monitored for metastasis. This procedure was repeated for up to 11 to 14 passages with from 2 to 19 mice being used at each passage (Tables 4.2, 4.3). At each transfer one of the tumour-bearing mice was selected at random as the tumour donor for the subsequent passage. At various transfer numbers (cf. Table 4.4) the tumours were returned to tissue culture, passaged several times *in vitro* and re-inoculated into animals as single cell suspensions.

The results of this series of experiments are presented in Table 4.2 and Table 4.3 and may be summarised as follows:-

UCT-Mel 3 was consistently metastatic from the start and remained so throughout the course of serial *in vivo* passaging. Metastases with this line were always seen but only if the primary lesion was allowed to reach a certain minimal size before excision (see below). The metastases used to start the transfer series (one from lung and one from the abdominal cavity) showed no significant differences either in metastatic potential or organotropic qualities.

It is of interest to note that an *in vitro* culture of UCT-Mel 3 that was taken at the 6th *in vivo* transfer and passaged *in vitro* 12 times retained tumorigenicity but appeared to have lost metastatic potential (Table 4.4). This interesting non-metastatic subline has been kept for further study and comparison with the parent.

UCT-Mel 5 started with a metastatic frequency of 14%. A lung deposit was taken from one of the three mice that developed secondaries and used to start the *in vivo* series. As can be seen from Table 4.2, all of the animals after the 7th transfer developed metastases.

An *in vitro* culture established from the tumour removed at the 5th passage gave rise to a line which caused metastatic tumours in 7/7 mice after 5 *in vitro* passages (Table 4.4).

Table 4.2 Incidence of metastases with repeated in vivo passage

		UCT-Mel 1	2	3	4	5
	Parent	1/17	3/26	35/35	1/10	3/22
Passage	1	1/13	0/2	4/4	0/3	2/3
	2	1/2	0/2	4/4	0/3	2/3
	3	6/14	0/2	3/3	1/3	9/14
	4	3/19	0/2	4/4	1/3	3/4
	5	4/19	0/2	3/3	2/2	4/5
	6	1/8	0/2	2/2	0/1	4/5
	7	1/12	0/2	2/2	0/2	2/3
	8	1/3	0/2	2/2	0/5	2/2
	9	0/2	0/2	3/3	0/2	2/2
	10	0/2	0/2	2/2	1/2	2/2
	11	0/2	0/2	2/2	0/2	
	12	0/2	0/2	2/2	0/2	
	13		0/2	2/2	0/2	
	14				0/2	

TABLE 4.3

METASTASES IN NUDE MICE

ORIGIN	NUMBER OF MICE	METASTASES (%)	SITE OF METASTASES
UCT-Mel 1_{Met}	86	21	Lung Kidney Gonad
UCT-Mel 2_{Met}	22	0	-
UCT-Mel 3_{Met} (abd)	25	80	Lung Kidney Gonad Adrenal
UCT-Mel 3_{Met} (lung)	40	100	Lung Kidney Gonad Adrenal
UCT-Mel 4_{Met}	28	18	Lung Kidney Adrenal
UCT-Mel 5_{Met}	50	78	Lung Kidney Gonad Stomach

TABLE 4.4

METASTASES OF HUMAN MALIGNANT MELANOMA IN NUDE MICE

CELL LINE/ ORIGIN	NO OF PASSAGE IN MOUSE/NO OF CELLS INOCULATED	TIME OF REMOVAL (DAYS)	NUMBER OF MICE	TUMOUR WT AT REMOVAL (GM)	LENGTH OF TIME TO METASTASES (DAYS)	METASTASES %	SITE
UCT-Mel 3	P6/1x10^{o}	78	2	0.4-0.8	131;133	0	-
(Lung met	P6/1x10^{6}	61;69;78	4	0.8-1.0	130;197	0	-
passaged s.c.)	P6/1x10^{6}	54;59;69	4	1.0-1.2	210;239;250;264	25	Abd.cav.
	P6/1x10^{6}	66;74	3	1.2-1.4	244;256	0	-
	P6/1x10^{6}	69	2	1.4-2.0	239;264	0	-
	P11/2x10^{6}	35;56;64	4	1.2-1.6	56;111;127	100	-Lung, Abd.cav.
		35;56;64	4	>2	56;59;167	75	-Lung, Abd.cav.
UCT-Mel 5	P5/2x10^{6}	56;63;65	3	0.6-1.0	162;169;183	100	Lung
(Lung met		56;65;83	4	1.0-1.4	148;160;162	100	Lung
passaged s.c)							

The original metastases from UCT-mel 5 were seen only in the lungs of 3 affected mice (Table 4.1). As the tendency to spread increased with in vivo transfer occasional metastasis to other organs was found (kidney, peritoneal cavity, stomach and uterus) but for the most part spread to these sites was rare. Lung remained the principal target organ for this tumour.

UCT-Mel 1-derived tumours gave rise to metastases in 1/17 animals when first studied (Table 4.1). the lung deposit that was selected to start the in vivo transfer series in this case gave rise to a subline that retained tumorigenicity but that appeared to have lost all tendency to spread (Table 4.2 and 4.3).

UCT-Mel 4 metastasized with a frequency of approximately 20%. The lung was the major site of metastatic spread.

Size of the primary tumour and metastasis.

Surgical approaches to the management of solid malignant neoplasms in man are firmly based upon the belief that the primary tumours should be excised at an "early" stage "before spread has occurred".

I felt that it should be possible, with the uniformly metastatic cell line, UCT-Mel 3, to give quantitative meaning to the term "early" in the above context by removing tumours at different sizes and/or after different periods of subcutaneous growth and noting the frequency of metastasis seen after resection.

The results of two such experiments are recorded in Table 4.5 and in Fig. 4.4a and 4.4b. The composite graph (Fig.4.5) of all the data shows, most clearly, the striking relationship between frequency of metastasis and size of the primary tumour at the time of resection. Spread did not occur if the primary tumour was removed before it had reached the size of 0.6g; when the tumour was allowed to grow to reach a size greater than 1.6 g, metastasis was invariably seen. Tumours of intermediate size metastasized with a correspondingly intermediate frequency. The data in Table 4.6 for the two individual

TABLE 4.5

METASTASES IN NUDE MICE

CELL LINE	NUMBER OF MICE	TUMOUR SIZE (gm)	METASTASES (%)	SITE OF METASTASES
UCT-Mel 3	13	0.2 - 0.6	0	-
UCT-Mel 3	8	0.6 - 0.8	50	Lung Kidney Liver Gonad
UCT-Mel 3	6	0.8 - 1.0	68	Lung Uterus Ovary
UCT-Mel 3	42	1.0 - 1.6	80	Lung Kidney Gonads
UCT-Mel 3	15	1.6 - 2.0	100	Lung Kidney Gonads

METASTASES OF HUMAN MALIGNANT MELANOMA IN NUDE MICE

CELL LINE/ ORIGIN	NUMBER OF CELLS	TIME OF REMOVAL (DAYS)	NUMBER OF MICE	TUMOUR WT AT REMOVAL (GM)	LENGTH OF TIME TO METASTASES (DAYS)	METASTASES %	SITE
UCT-Mel 3	$1.0x10^6$	43;59	6	0.2-0.4	118(2);215(2);396	0	-
(Parent cells)	$1.0x10^6$	59;63	5	0.4-0.6	130;154;224;223;173	0	-
	$1.0x10^6$	59;92;150;171	5	0.6-0.8	141;154;175;250;239	50	Lung,Uterus Ovary,Kidney
	$1.0x10^6$	80;93;193	6	0.8-1.0	136;166;175;113;126;134	100	Lung,Uterus, Ovary,Kidney
	$1.0x10^6$	80;93;107	4	1.0-1.2	100;141;181;130	100	Lung,Uterus Ovary,Kidney
	$1.0x10^6$	82;107;176	4	1.2-2.0	132;210	100	Lung,Uterus Ovary,Kidney
	$1.0x10^6$	82;93	3	>2	130;136;148	100	Lung, Abd.cav. Ovary
	$1.8x10^6$	66	1	0.2-0.4	156	0	
	$1.8x10^6$	45;48;57	4	0.4-0.6	211;227;152;235	25	Lung,Kidney, Gonads
	$1.8x10^6$	45;48;57	9	0.6-1.0	116;12;144;186;167;175	24	Kidney,Gonads, Intestine,Liver
	$1.8x10^6$	48;57	3	1.0-1.2	111;121;198	66	Lung,Gonads, Kidney
	$1.8x10^6$	57;66	2	1.2-1.4	114;121	100	Kidney,Gonads, Lung,Liver
	$1.8x10^6$	66	7	>2	106;118;143;156;169	100	Lung,Kidney, Gonads,Submax-illary gland
	$5.0x10^6$	67	3	>2	116;119	100	Lungs, Abd.cav.

FIGURES 4.4a and 4.4b

Figure 4.4a

Relationship between UCT-Mel 3 tumour size, length of time of tumour in the mouse and metastasis

Mice were inoculated subcutaneously with 10^6 to $5x10^6$ UCT-Mel 3 cells (*in vitro* passage numbers 69' or 87'). Tumours of various sizes were removed at time 0. Mice were then observed and were sacrificed once weight loss occurred (this was found to be a reliable indicator of metastatic spread). Metastatic spread was observed in all animals and was documented by autopsy and histological examination of affected tissues.

As can be noted from this figure the length of time the tumour was present in its host did not affect the incidence of metastasis. The size of the primary tumour at the time of its removal determined the incidence of metastatic spread.

(▲-----▲) metastasis (63)

Figure 4.4b

Relationship between UCT-Mel 3 tumour size, length of time of tumour in the mouse and metastasis

Mice were inoculated subcutaneously with 10^6 to $5x10^6$ UCT-Mel 3 cells (*in vitro* passage number 69' or 87'). Tumours of various sizes were removed at time 0. No metastases were observed in these animals.

As can be noted from this figure the length of time the tumour was present in its host did not contribute to the incidence of metastasis. The size of the primary tumour at the time of its removal determined the incidence of metastasis (see Fig. 4.4a).

(■———■) No metastasis (21).

Figure 4.4a

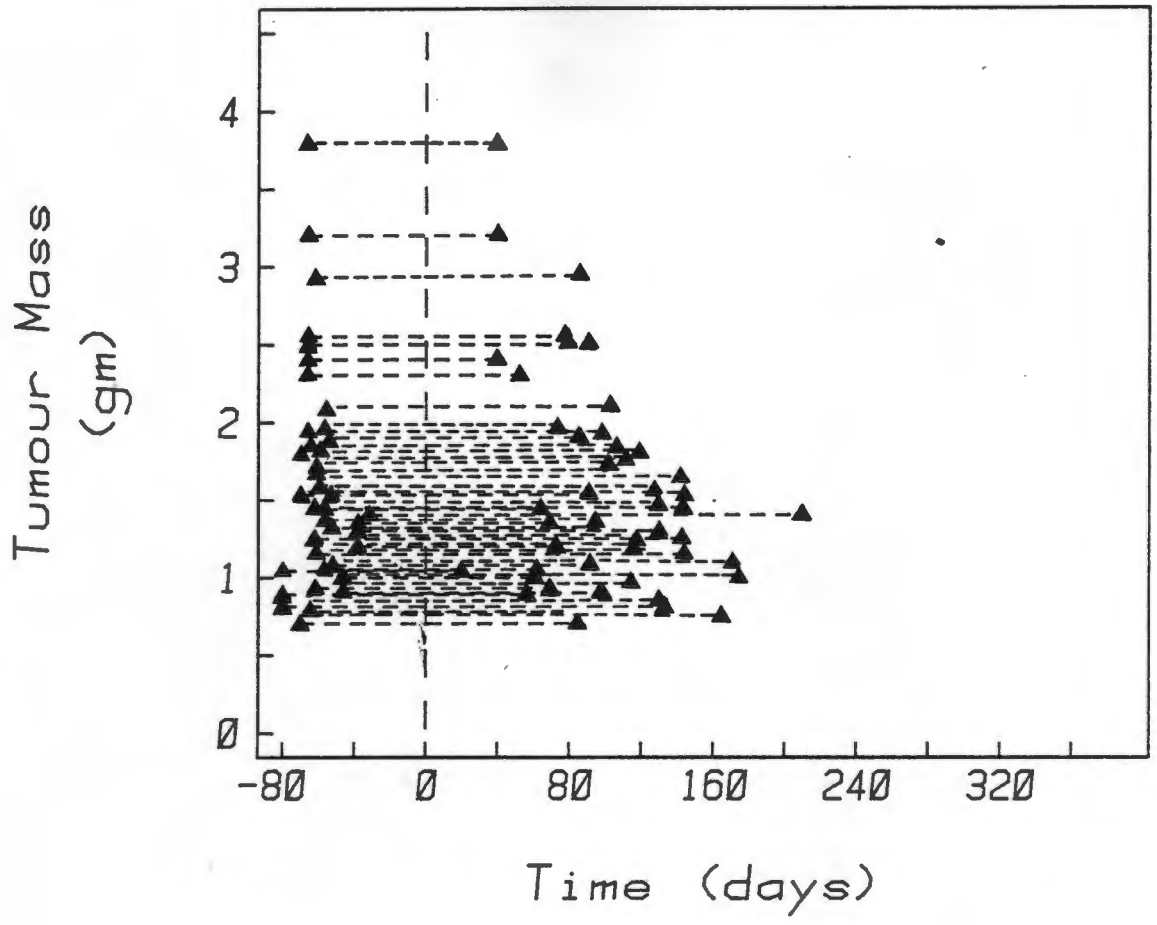


Figure 4.4b

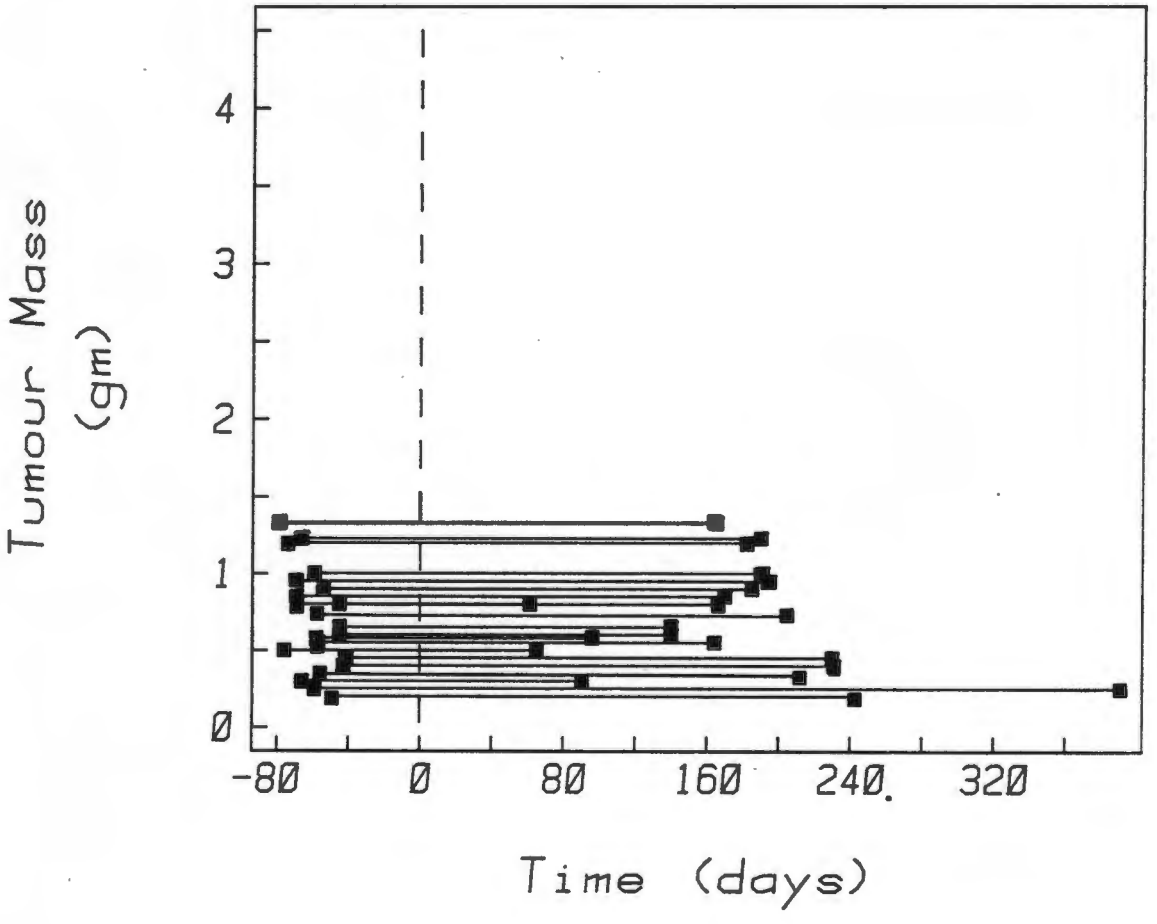


FIGURE 4.5

Figure 4.5

Relationship between UCT-Mel 3 tumour size and metastasis in the nude mouse

Mice were inoculated subcutaneously with 10^6 to $5x10^6$ UCT-Mel 3 cells (*in vitro* passage numbers 69' or 87'). Tumours of various sizes were removed. Mice were then observed and were sacrificed once weight loss occurred (this was found to be a reliable indicator of metastatic spread). Metastatic spread was documented by autopsy and histological examination of affected tissues.

Note the following:-

(a) If the tumour was less than 0.6 gm at the time of surgery no metastasis occurred (13 mice).

(b) If the tumour was between 0.6 - 1.0 gm when removed 60% of the mice developed metastatic malignant melanoma (14 mice).

(c) If the tumour was between 1.0 - 1.6 gm when removed 85% of the mice developed metastasis (42 mice).

(d) If the tumour was greater than 1.6 gm at the time of removal all mice developed metastasis (15 mice).

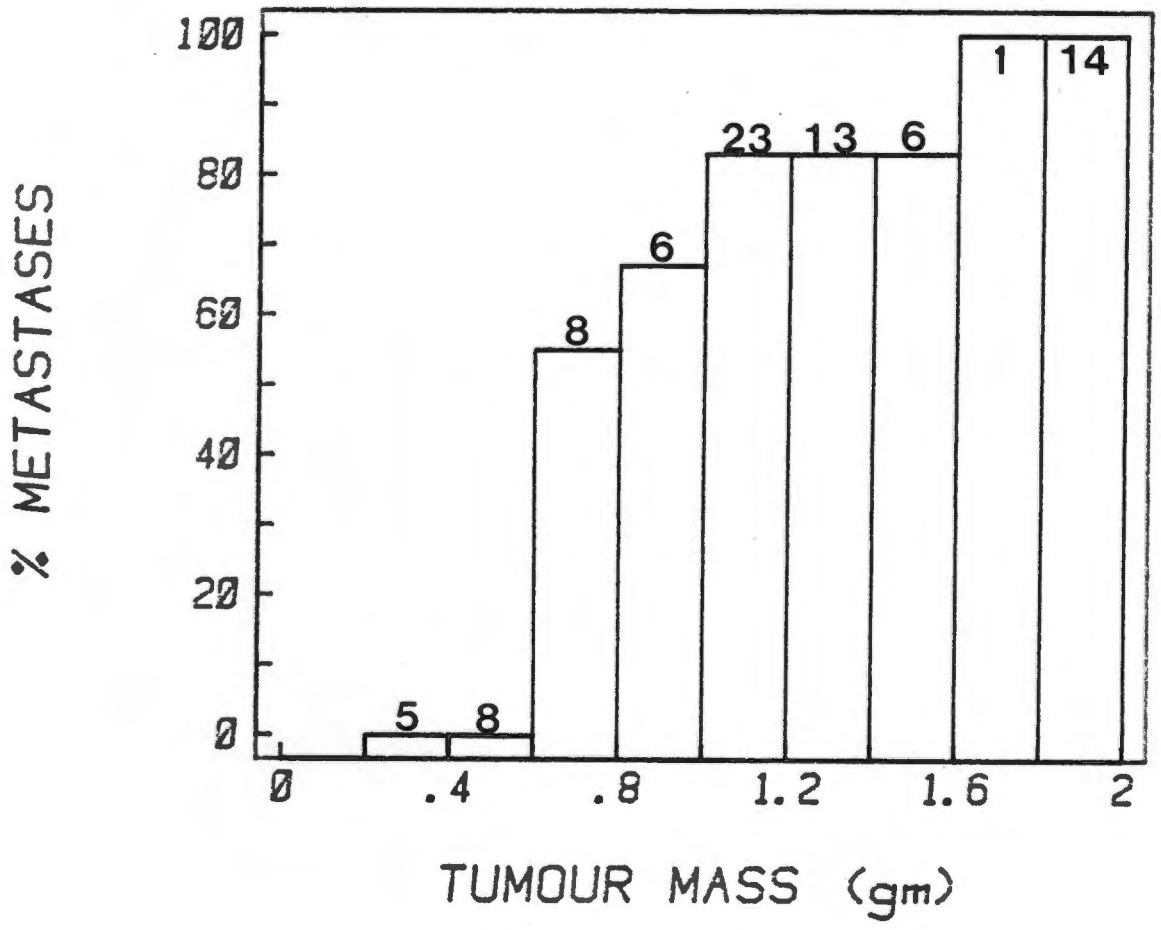

% METASTASES
100
80
60
40
20
0
5
8
8
6
23
13
6
1
14
0
.4
.8
1.2
1.6
2
TUMOUR MASS (gm)

Figure 4.5

experiments showed that the effect of mass upon spread could be demonstrated reproducibly.

The frequency with which metastasis occurred was related to the size attained by the tumours and not to the period of subcutaneous growth (Figs. 4.4a and 4.4b).

Although the number of animals studied is as yet too small to warrant emphasis it is of interest to note that, in the case of UCT-Mel 5, the parent line showed an inverse relationship between tumour size and metastatic frequency (Fig.4.6). This tendency for large primary tumours not to be associated with secondary disease was no longer observed with uniformly metastatic sublines derived from repeated in vivo passage (Fig. 4.7 and Appendix Table A.13).

Time taken for metastases to develop.

I have previously observed (Fig. 10, Chapter 3) that, in my experience, subcutaneous tumorigenic inocula of human melanoma cells invariably produced tumours in 84 days or less and that 95% of tumours that were going to develop did so within 42 days. In certain respects the deposition of a micrometastasis in a target organ may be seen as being similar to the deposition of a tumorigenic inoculum into the subcutaneous tissues, so it was of interest to see how the time taken for metastasis to manifest after excision of the primary compared with the time taken for subcutaneous tumours to develop.

Data accumulated from many observations of UCT-Mel 3-derived tumours (Fig. 4.8) showed that, taking the onset of weight loss as the first sign of established metastatic disease, 89% of metastases declared themselves in 140 days of removing the primary tumour and virtually all within 160 days. One case required observation for 210 days before metastases were seen.

Although there was some tendency for metastases to develop sooner after the exicision of large tumours than after the removal of smaller masses, this inverse relationship was by no means uniform (Fig. 4.9).

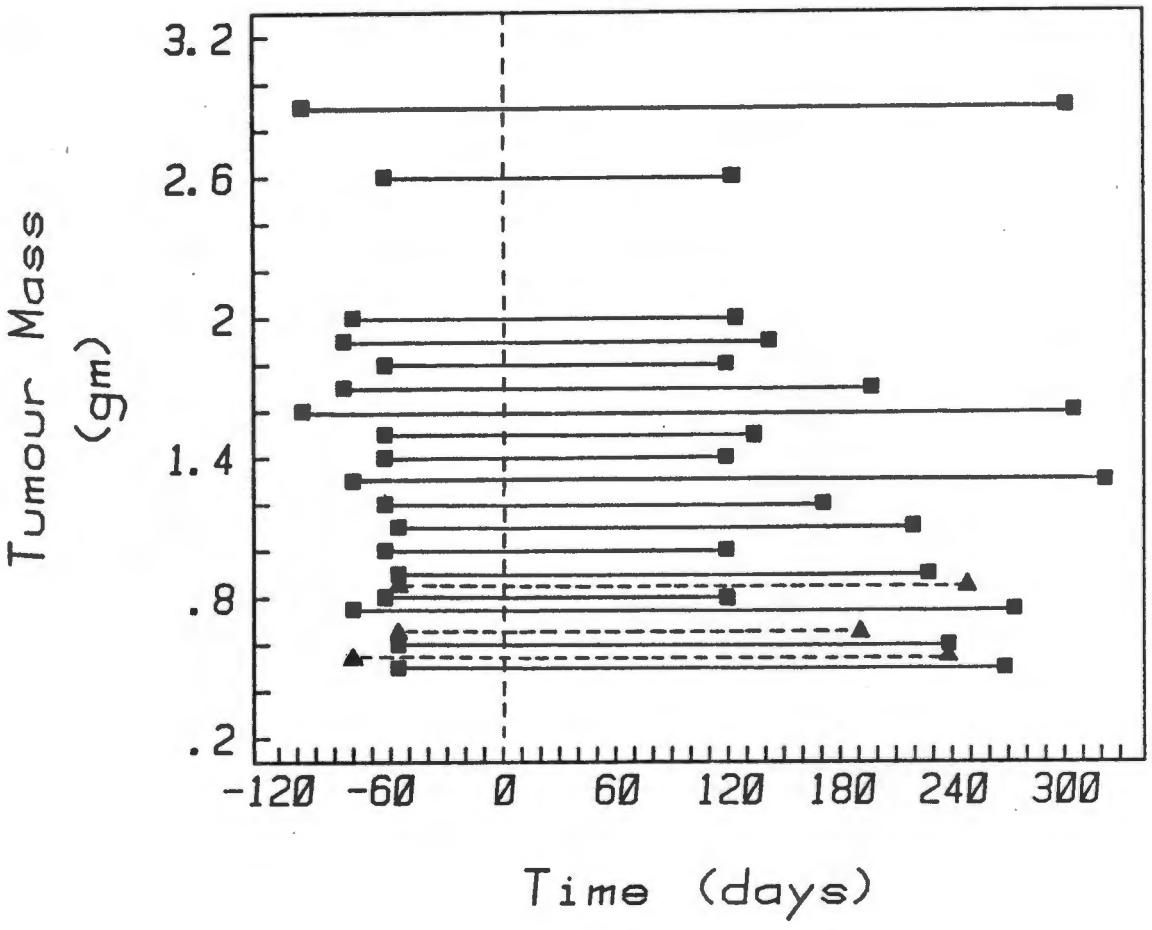


Figure 4.6

Relationship between UCT-Mel 5 tumour size in the nude mouse and metastasis

Mice were inoculated subcutaneously with 10^6 or $5x10^6$ parent UCT-Mel 5 cells (in vitro passage number 27'). Tumours of various sizes were removed at time 0. Metastatic spread was documented by autopsy and histological examination of affected tissues.

As can be noted from this figure the size of the primary tumour at the time of its removal did not determine the incidence of metastasis in these animals.

(▲----▲) metastasis (3)

(■——■) no metastasis (18)

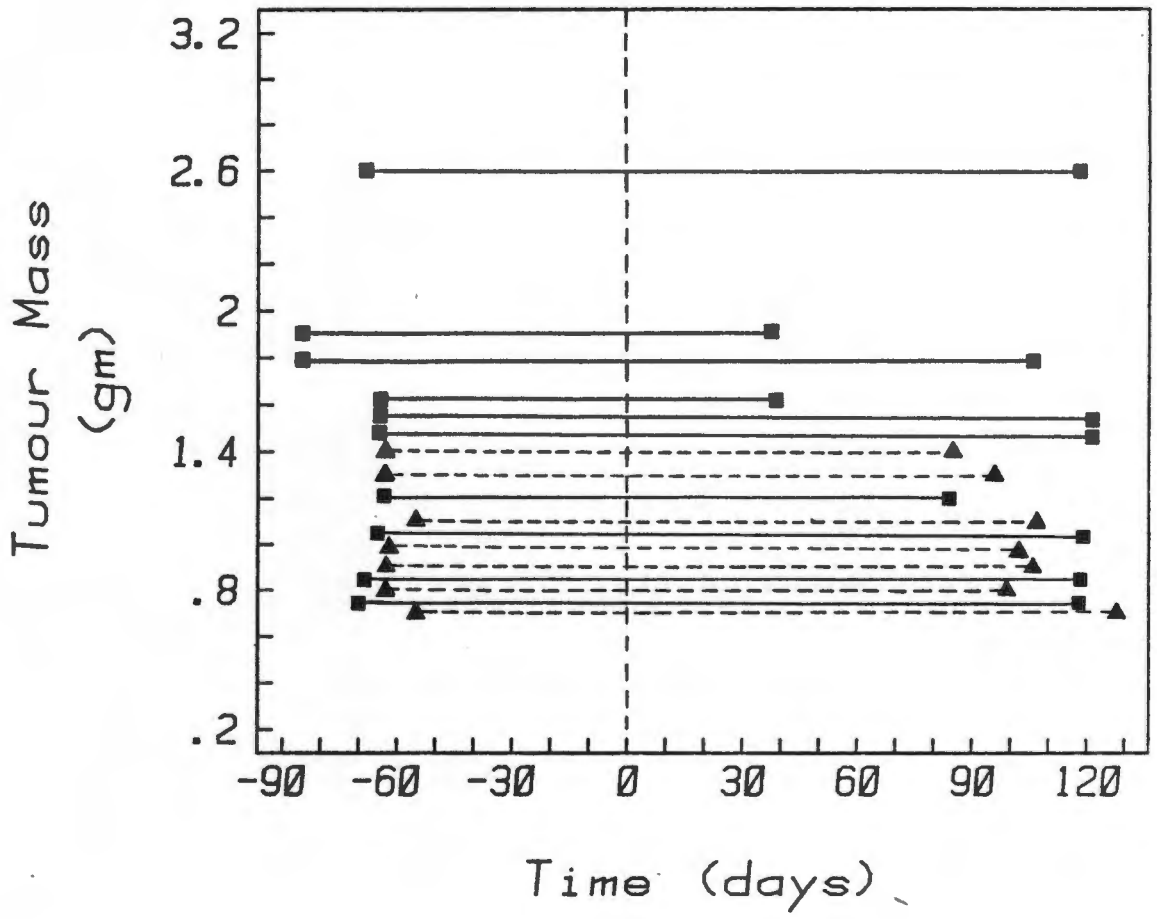


Figure 4.7

Relationship between UCT-Mel 5 tumour size in the nude mouse and metastasis

Mice were inoculated subcutaneously with $2x10^6$ UCT-Mel 5 parent cells (*in vitro* passage number 27') or $2x10^6$ UCT-Mel 5 metastatic subline (*in vivo* passage 5' and *in vitro* passage number 5'). Tumours of various sizes were removed at time 0. Metastatic spread was documented by autopsy and histological examination of affected tissues.

As can be noted from the graph, the metastatic subline originating from a lung deposit, metastatised in all cases (7/7), whereas the parent tumours removed in the same weight range, did not produce metastasis at all (0/10).

(▲----▲) metastases (7/7)

(■——■) no metastases (0/10)

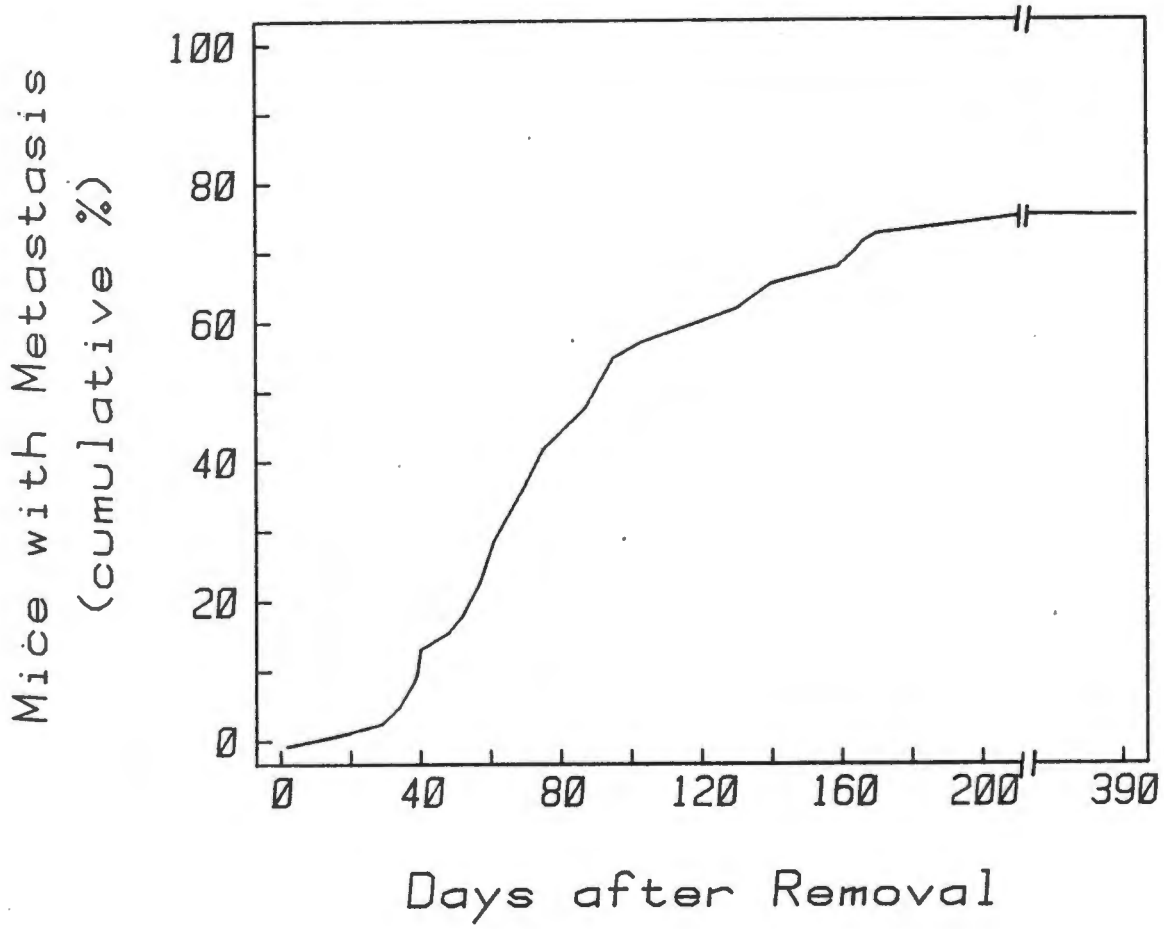


Figure 4.8

Incidence of metastases as function of time following UCT-Mel 3 tumour removal

Eighty four mice were inoculated subcutaneously with 10^6, 1.8×10^6 or 5×10^6 UCT-Mel 3 cells(*in vitro* passage numbers 69' or 87'). Primary tumours of different sizes were removed on day 0. The graph represents a plot of cumulative metastases appearance as a function of time following the removal of the primary tumour. The onset of weight loss was taken as the first sign of metastatic disease. Metastases occurred in 63 mice. Eighty nine per cent of these metastases were evident 140 days following removal of the primary tumour. 62/63 mice had metastatic spread by 160 days and the remaining mouse by 210 days.

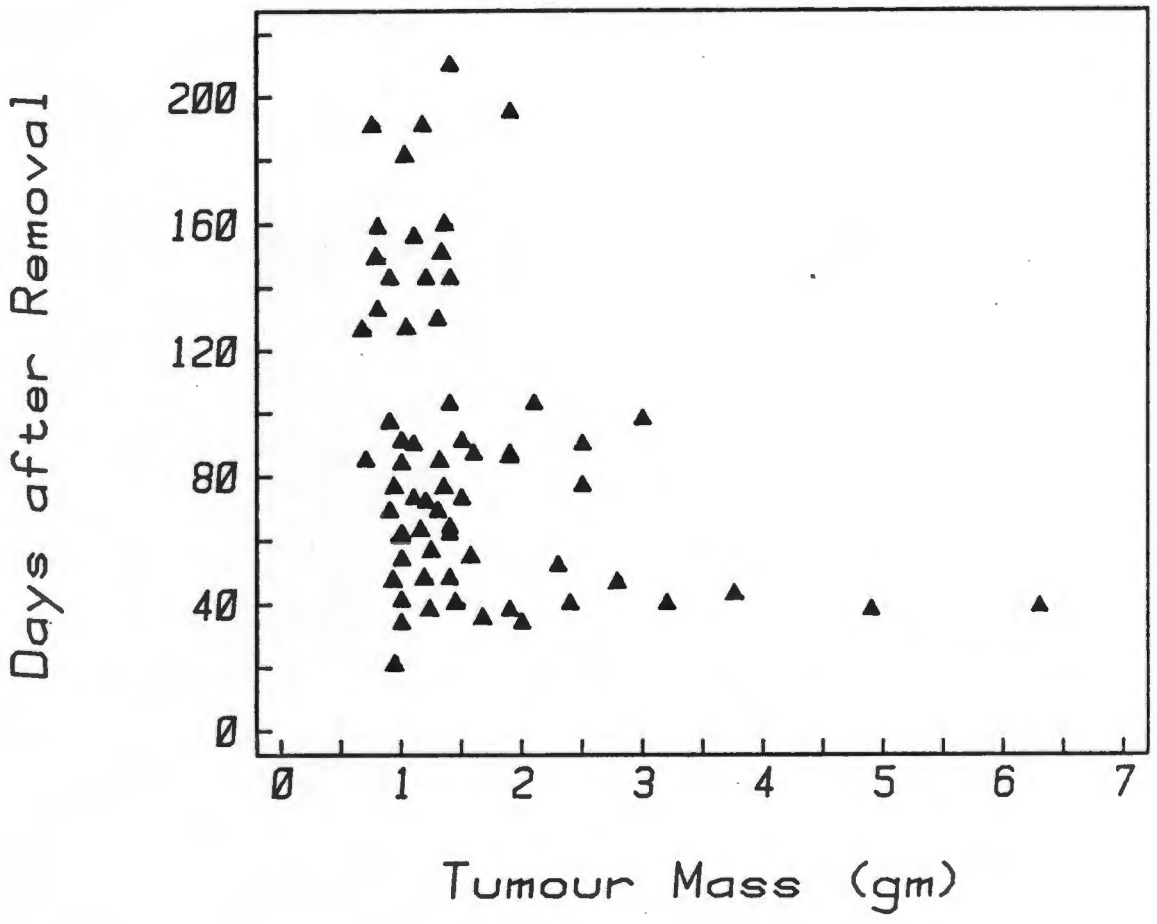


Figure 4.9

The relationship between the mass of the primary tumour and the length of time to metastasis

Sixty three mice were inoculated subcutaneously with 10^6, 1.8×10^6, 2×10^6 or 5×10^6 UCT-Mel 3 cells (in vitro passage numbers 69' or 87') on day 0. Tumours of various sizes were removed and the tumour mass at the time of removal plotted as a function of the time taken for metastases to develop. Tumours were removed at a size which ensured metastatic spread (see Fig. 4.5).

Note that metastases which occurred following the removal of smaller tumours were found over a fairly extensive time period, namely 20-210 days after removal of the primary tumour. Metastases following removal of larger tumours were always detected within 45 days of removal of the primary tumour.

Target organs

As I have indicated earlier, repeated *in vivo* passage of tumours derived from a secondary deposit from UCT-Mel 1 did not appreciably affect the metastatic potential of the cells in terms of the frequency with which they gave rise to secondary deposits. In the 18 mice in which metastases occurred lungs were the preferred site in approximately half the animals with the abdomen being involved in about 20% of mice (Fig. 4.10).

The metastatic deposit from UCT-Mel 2 did not give rise to metastases in any of the mice inoculated (Table 4.2).

UCT-Mel 3 spread consistently to many organs with the lung and abdomen being preferred sites (Figs. 4.11, 4.12, 4.13).

Fig. 4.14 shows a number of metastatic focus in the lung of a mouse, that was inoculated intravenously with 2×10^6 UCT-Mel 3 cells.

The incidence of metastases did not increase with passage through mice in the case of UCT-Mel 4 (Table 4.2). Five mice in this study developed metastases, all of which involved the lungs (Fig. 4.15).

As already mentioned UCT-Mel 5 started with a metastatic frequency of 14% and increased to 100% with serial passage through mice (Table 4.2). Metastases from this line were almost exclusively confined to the lungs (Fig. 4.16 and 4.17).

Cellular comparison

The availability of melanoma cell lines with demonstrably different capacities for forming metastatic tumours enabled me to compare them with respect to certain attributes that may be relevant to the metastatic phenotype.

It is interesting to note that the rate of plasminogen activator release by parent cells cultured *in vitro* was much greater than that of metastatic isolates (Table 4.7). I also found that the kinetics of growth *in vitro* or *in vivo* bore little relationship to the metastatic potential of the cells.

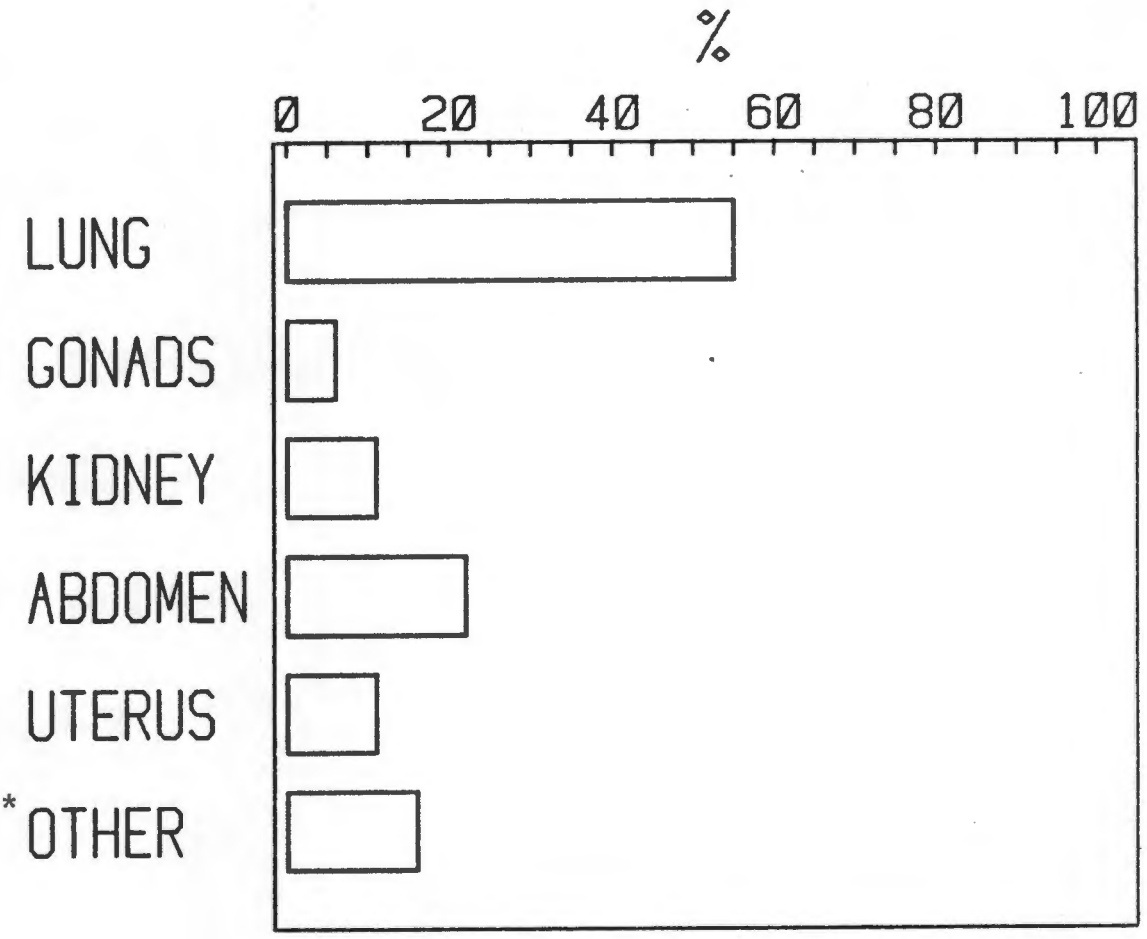


Figure 4.10

The location of metastatic deposits following either the subcutaneous inoculation of UCT-Mel 1 metastatic deposits from the lung

Eighty six mice were inoculated subcutaneously with either 10^6 or $5x10^6$ UCT-Mel 1 cells (*in vitro* passage number 65') or with metastatic explants removed from a lung deposit which had been passaged several times (12) in nude mice. Nineteen mice developed metastasis, primarily of the lung but deposits in abdomen, kidney, uterus, gonads, intestine and lymph node were also found.

*Intestine (1); Lymph node (1).

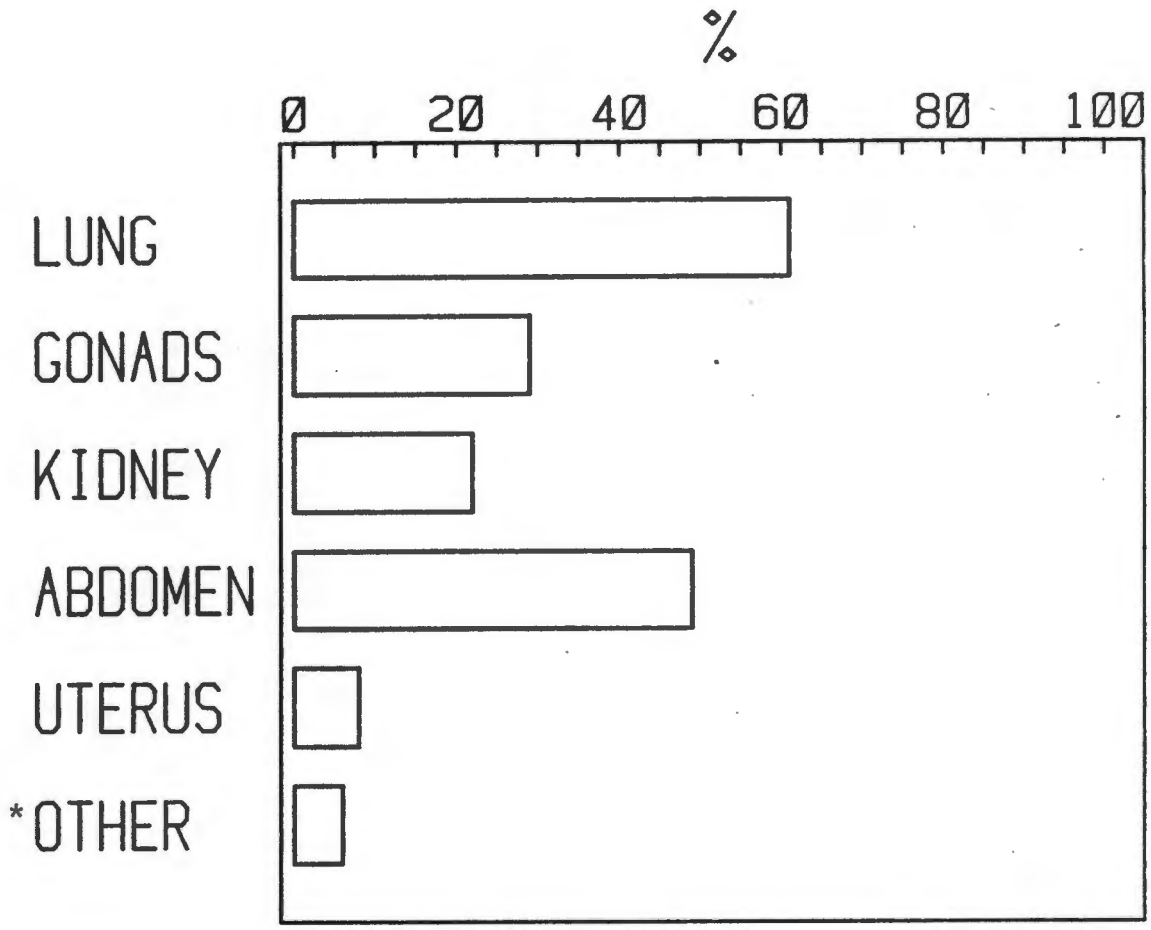


Figure 4.11

The location of metastatic deposits following subcutaneous inoculation of UCT-Mel 3 cells

Fifty one mice were inoculated subcutaneously with 10^6 to $5x10^6$ UCT-Mel 3 parent cells (in vitro passage numbers 69' and 87'). All mice developed metastases which were widespread and involved primarily the lungs, abdomen, gonads, kidney and uterus. However metastatic deposits were also found in the lymph node (1) and the submaxillary gland (1).

*Submaxillary gland (1); Intestine (1); Lymph node (1).

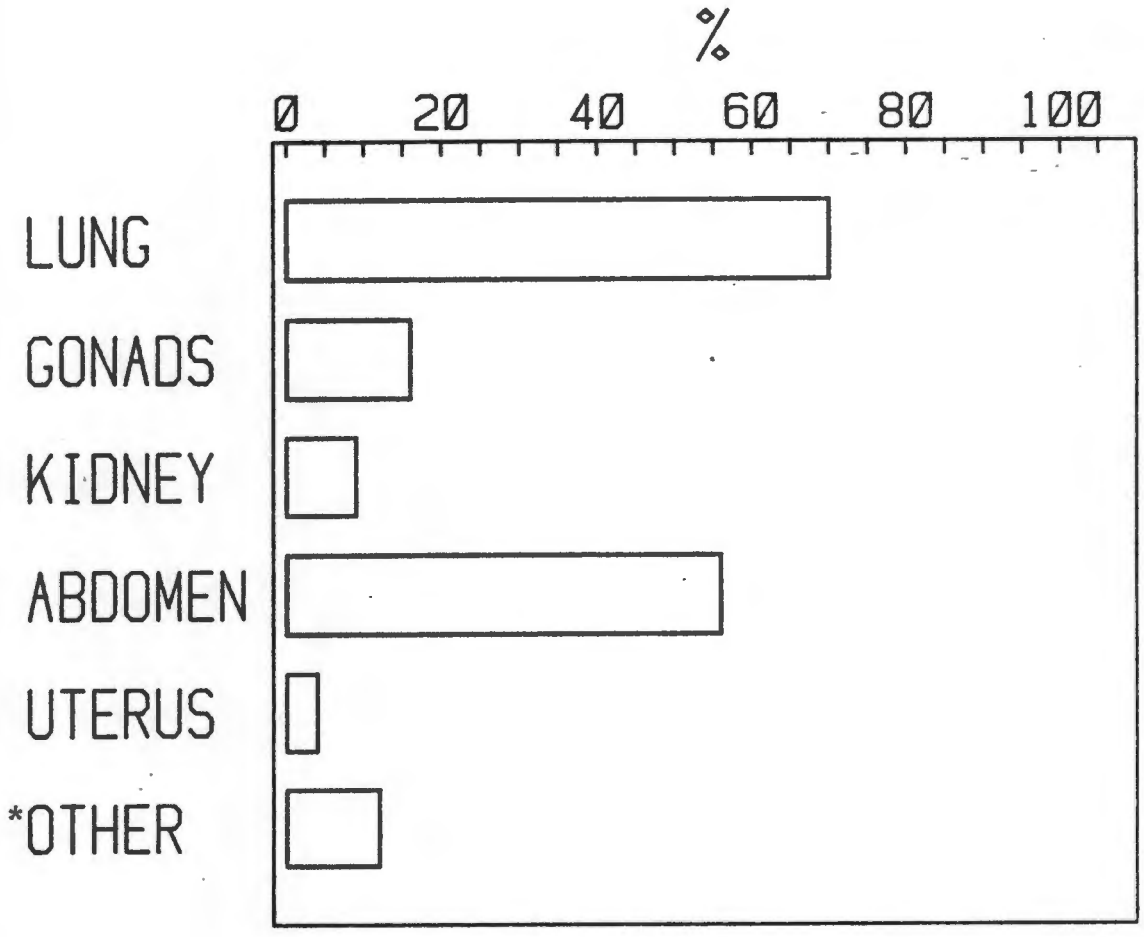


Figure 4.12

The location of metastatic deposits following subcutaneous implantation of a UCT-Mel 3 lung metastasis

Thirty five mice were implanted subcutaneously with metastatic explants originating from a lung deposit which was passaged several times (13) through nude mice. All mice developed metastases primarily of the lung and abdomen, but deposits in the gonads, kidney, uterus, eye, lymph node, bladder and adrenal gland was also found.

*Eye (1); Lymph node (1); Bladder (1); Adrenal gland (1).

FIGURE 4.13

Figure 4.13

Widespread metastasis of UCT-Mel 3 in abdominal cavity of the nude mouse

Mice were inoculated subcutaneously with 5×10^6 UCT-Mel 3 cells (in vitro passsage number 69'). The primary tumour was removed 66 days after inoculation, once a size of approximately 2300mm^3 had been reached. the mouse was sacrificed once weight loss occurred (this was found to be a reliable indicator of metastasic spread).

Metastatic spread was observed throughout the abdominal cavity involving the majority of organs.

This figure illustrates:-

(a) Kidney metastasis.

(b) Metastasis in the mesentery.

(c) Large metastatic tumour involving the left ovary.

(d) Lymph node metastasis.

(e) Metastatic focus involving the bladder.

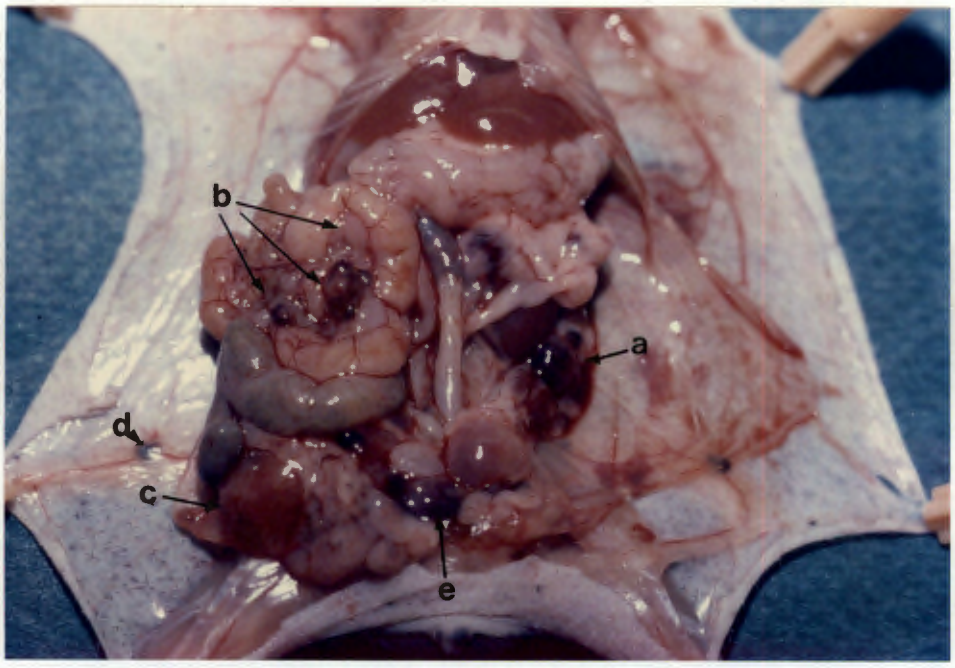


Figure 4.13

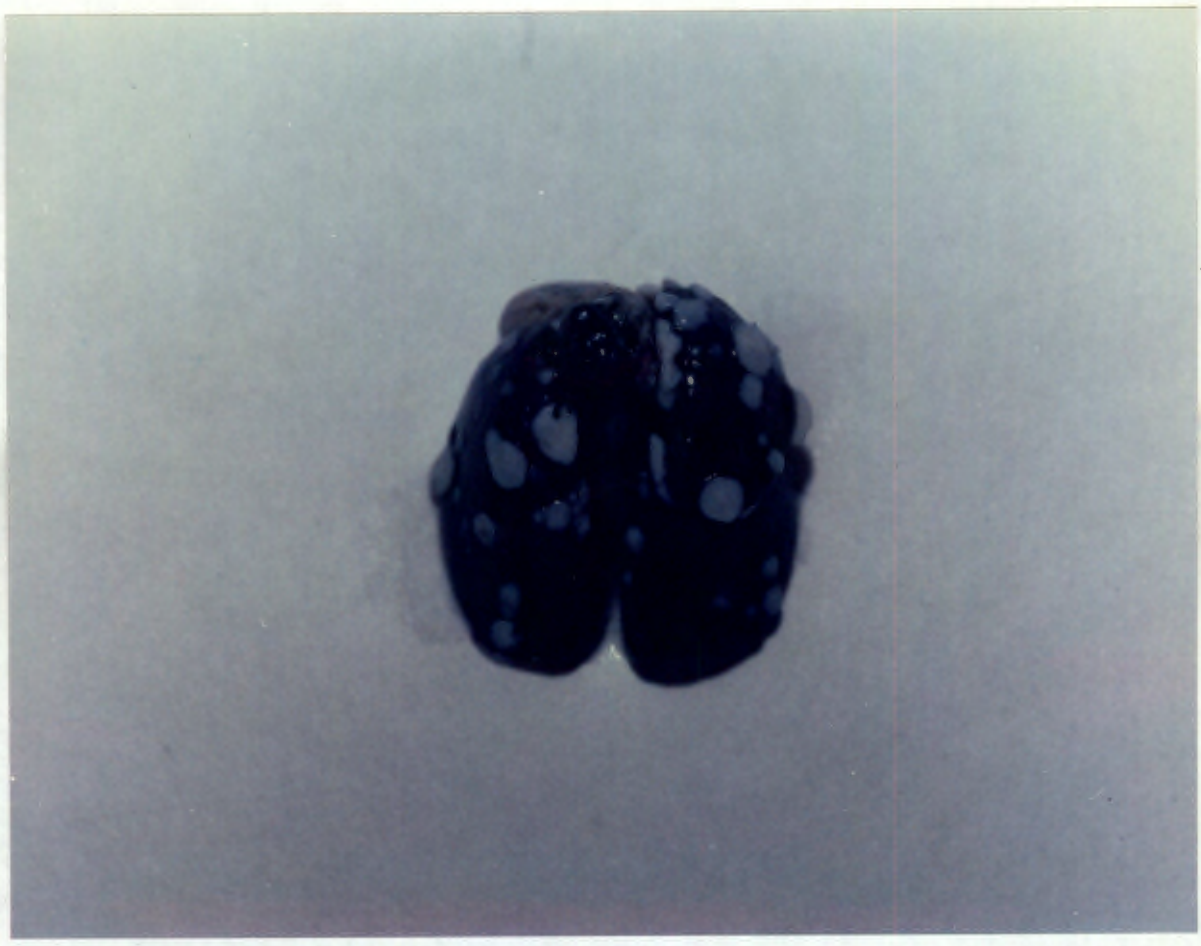


Figure 4.14

Lung metastasis of UCT-Mel 3 in nude mice.

A mouse was inoculated intravenously in the tail vein with 2×10^6 UCT-Mel 3 cells (69'). After 6 weeks the mouse was sacrificed and the lungs infiltrated with Indian ink and fixed in formalin as described in the text.

Note from the picture that multiple metastases are visible as unstained white nodules in the lungs.

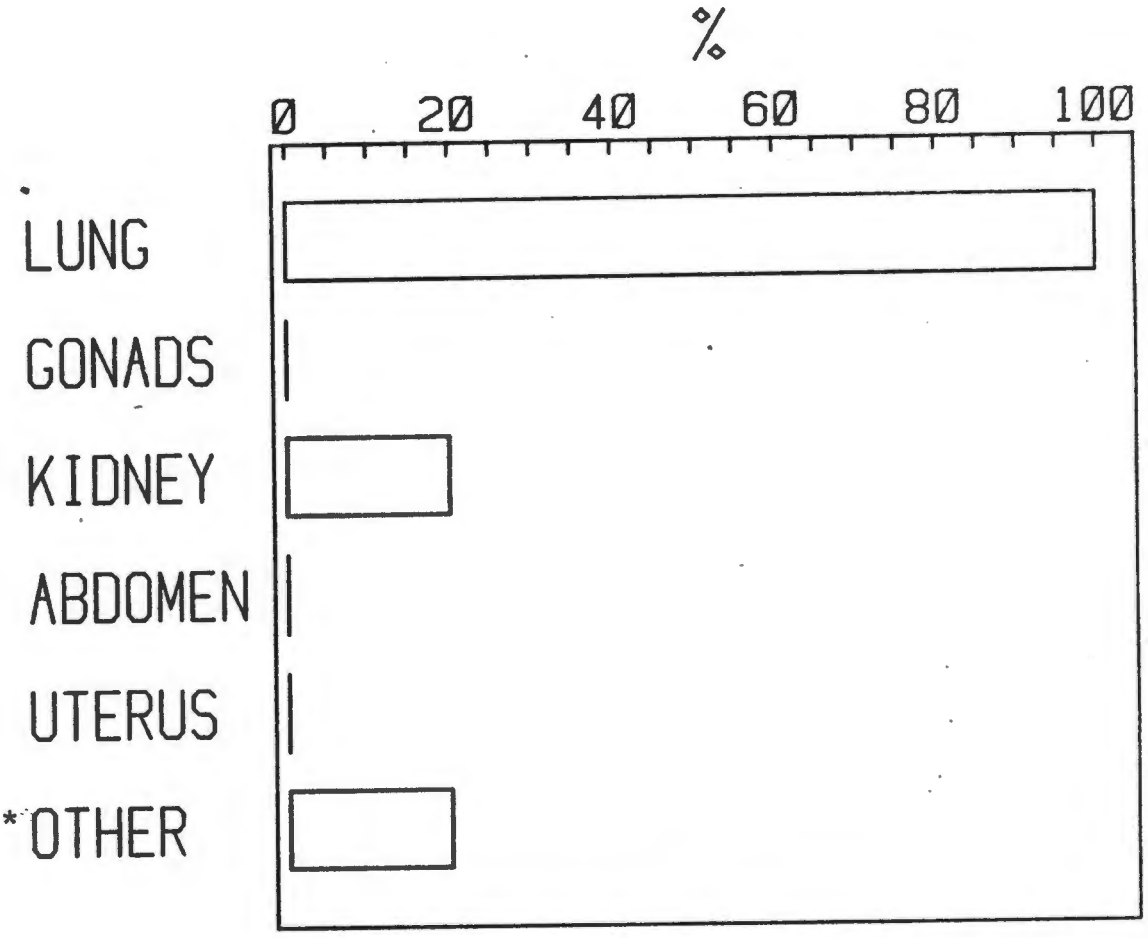


Figure 4.15

The location of metastatic deposits following subcutaneous implantation of a lung metastasis obtained from UCT-Mel 4

A lung metastasis from a mouse inoculated with UCT-Mel 4 cells was removed and passaged 14 times through nude mice. Thirty four mice received subcutaneous implants of this UCT-Mel 4 *in vivo* deposit. Five mice developed metastasis, all of which were found in the lung. Metastatic deposits were also found in the kidney (1 mouse) and the adrenal gland (1 mouse).

* Adrenal gland (1).

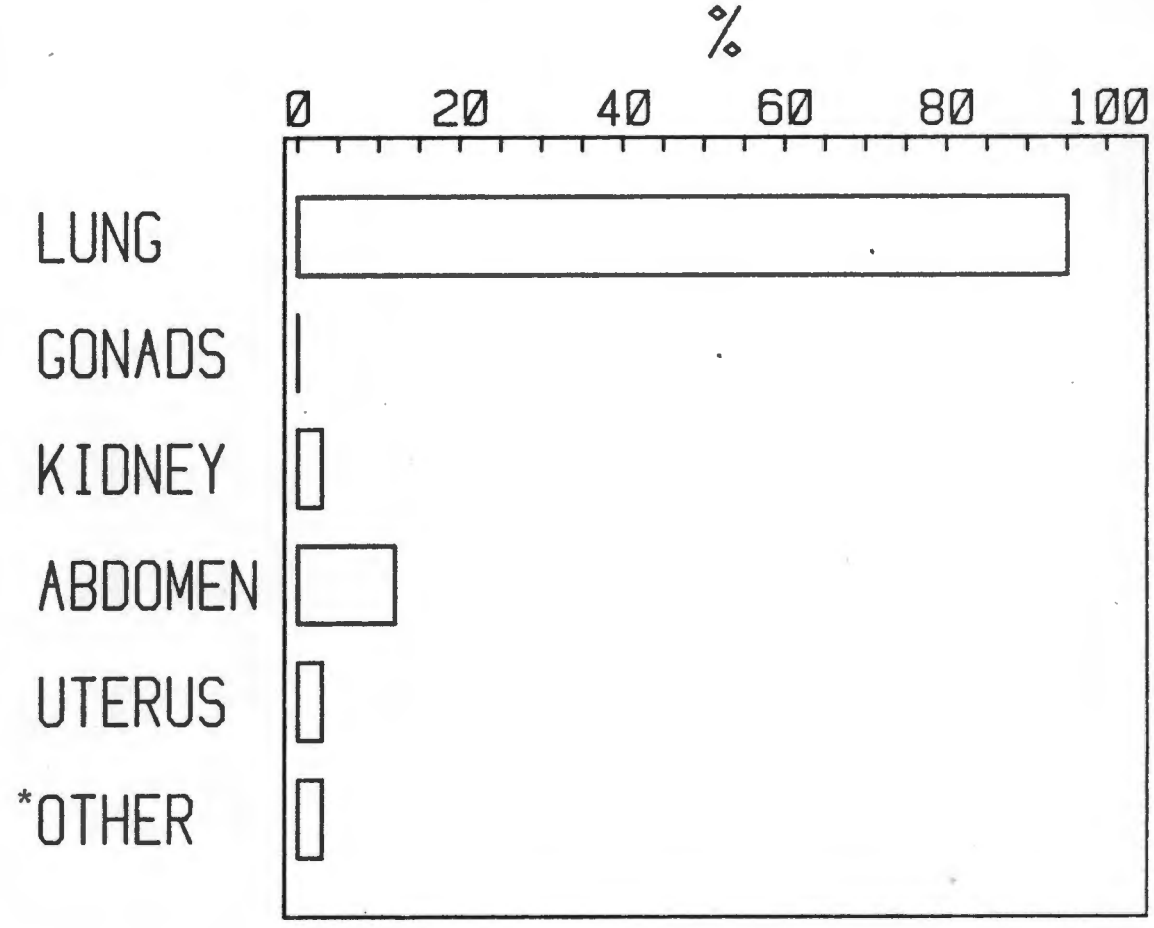


Figure 4.16

The location of metastatic deposits following subcutaneous inoculation of UCT-Mel 5 cells

Fifty mice were inoculated subcutaneously with either 10^6 UCT-Mel 5 parent cells (*in vitro* passage number 27') or with 2×10^6 UCT-Mel 5 metastatic subline cells (*in vivo* passage number 5 and *in vitro* passage number 5'). Thirty nine mice developed metastasis almost exclusively of the lung, but deposits in kidney, abdomen, uterus and stomach were also found.

* Stomach (1).

Figure 4.17

Lung metastasis of UCT Mel 5 in nude mice.

A mouse was inoculated subcutaneously with 10^6 UCT-Mel 5 cells (27'). The primary tumour was removed once a size of approximately 2000mm^3 was reached. One hundred and twelve days after the tumour was removed the mouse was sacrificed and the lungs infiltrated with Indian ink and fixed in formalin.

As shown in the picture four large metastatic foci are evident as unstained areas in the lung.

In the case of UCT-Mel 5, the highly metastatic variant derived by the repeated *in vivo* passage had a shorter doubling time *in vitro* (33 hr)than the original parent (58 hr) (Table 4.7). In the case of UCT-Mel 3 the highly metastatic subline had a much longer *in vitro* doubling time (86 hr) than the equally metastatic parent cells (58 hr)(Appendix Figs. A.14 and A.15).

Table 4.7

Cell line	T12 in vitro	PA $u/10^6$ cells/24hr	Metastases
UCT-Mel 3			
Parent	58	9.2	100%
Subline	86	0.2	100%
UCT-Mel 5			
Parent	58	3.8	14%
Subline	33	0.9	100%

DISCUSSION

The results that I report in this chapter show that nude mice bearing xenografted human tumours provide useful experimental models for the study of tumour metastasis. Five of the six tumorigenic melanoma cell lines that I have studied gave rise to metastatic tumours and in at least two cases (UCT-Mel 3 and 5) these could be used to document interesting features of the metastatic process.

My experience runs counter to that of most other reports in the literature since these have been generally unenthusiastic about the nude mouse as a suitable host for the study of dissemination of human tumours. In excellent review of this subject, Sordat, Ueyama and Fogh (1982) concluded that nude mice may serve as useful experimental hosts for metastastizing tumours derived from a limited number of cell lines such as the MeWo or the SK-Mel 3 human melanomas, but their figures nevertheless showed that dissemination during the primary tumour-bearing life of the animal was an unusual event.

By excising the primary tumour I was able to prolong the life of the animal and so provide time for metastases to develop and declare themselves. The success that I have had is largely attributable to this strategem. It is quite clear from the literature, that investigators studying transplantable murine tumours were well aware of the fact that metastasis took time to become manifest and that their appearance was forestalled by the death of the host from the growth of the primary tumour (Reid et al.,1978; Wilson et al., 1984). It is somewhat surprising, therefore, that, with the notable exception of workers such as Sordat, Schatten and Ketcham (Ozzello and Sordat, 1980; Schatten, 1958; Ketcham et al., 1959) this knowledge appears not to have been applied as fully as it might have been to xenograft systems.

The fact that the primary tumours had to be resected to demonstrate metastases immediately raises a question that is, in my opinion, central to the

understanding and preventive management of metastatic disease: is there a minimal size that a tumour must attain before metastasis will occur? In other words, does dissemination occur at an early stage in the natural history of a malignant neoplasm or is it rather a complication of late disease?

I believe that, in one case at least, my experiments have given an unequivocal answer to this question. Mice bearing tumours derived from UCT-Mel 3 cells survived without metastases if the tumours were excised before they had grown to exceed a mass of 0.6g. If the tumours were left until they were larger than 1.2g, all of the hosts developed disseminated disease. During the course of one effective doubling time, therefore, a prognostic transition took place: from 100% cure to 100% failure.

I have, unfortunately, no definitive explanation to offer for this dramatic effect of tumour size upon the outcome of the disease. It is, none the less, worth considering possible mechanisms - if only for the value that such speculation may have for the formulation of further experiments that need to be done.

In the first place, it is possible that the effect of size upon metastasis may be due to the fact that early primary tumours contained a genetically unstable population of non-metastasizing cells that progressed to the invasive and the metastatic phenotype. It is well known that the characteristics of tumours may change during the course of their growth so that they assume a more aggressive or "malignant" aspect. First studied systematically by Foulds (1958) approximately 30 years ago, tumour progression may occur as an abrupt, step-wise change over a fairly short period of time (Nowell, 1976) and I assume, therefore, that one population doubling might allow sufficient time for metastatizing clones within the neoplastic population to evolve. Some of the most convincing animal studies on tumour progression have recorded the spontaneous development of resistance to cytotoxic therapy (Skippers, 1983) in experimental tumours. These authors showed that drug treatment of

small tumours was usually curative whereas failure was frequent if the mass was allowed to attain a large size before starting therapy. In these experiments, mutations to a cytotoxic-resistant phenotype were frequent in tumours that were an order of magnitude larger than the sensitive counterparts, whereas my results showed a much smaller mass difference between confined and disseminated tumours. It is for this reason more than any other that I am disinclined to accept progression as a likely cause of the acquisition of metastatic behaviour in UCT-Mel 3. The appropriate experiments to investigate this possibility, however, have not been done and we should not dismiss this explanation until the results of these are available. Clearly it is necessary to know whether or not metastases are derived from metastatic subclones. This question would be answered affirmatively if it could be shown that subcutaneous tumours established from metastatic deposits disseminated at an earlier stage - i.e. before they had attained the mass of 0.6g. Furthermore, if the transition from localized to metastatic were invariably found to take place between 0.6 and 1.6 g (as it was in the two experiments that I performed) this would argue against progression since it is unlikely that random clonal change would consistently produce its qualitative effects within such a narrow mass range.

The second explanation that may be offered for the size affect might be that, since the process of metastasis depends upon the rate of entry of tumour cells into the circulation (Sordat 1977; Warenius et al.,1980) it would depend upon vascularization of the xenograft and the generation of a murine stroma. It may well be that the relative vascularity of the UCT-Mel 3 derived tumours (i.e. blood flow/gram tissue) is a direct function of size, and that at a size greater than 1.0g a critical metastatic flow is established. Methods, such as those using radioactive microspheres (Domenech et al., 1969) are available for measuring regional vascularity <u>in vivo</u> and the application of these might well shed light on this possibility.

Thirdly, the stroma may also be important if, as suggested by the work of De Vore et al., (1980) the fibroblastic and collagenous matrix around the tumour cells were to have anti-collagenolytic activity that matched, in qualitative terms, collagenase production by a small tumour but was unable to inhibit the enzyme produced by mass of twice the size. In the latter case free collagenase would be available to mediate invasion. This notion is supported by the work of Liotta (Liotta et al., 1981) who has produced convincing evidence to suggest a role for type IV collagenase in tumour spread. Measurement of collagenase activity released by UCT-Mel 3 cells and of the inhibition of this action by murine fibroblasts isolated from the stroma of the tumour might provide a useful approach to the investigation of this possibility. In similar vein, work from this laboratory has shown that fibroblasts, by binding and degrading tissue plasminogen activator (Hoal et al., 1983), effectively reduce the concentration of this enzyme. If, as so elegantly demonstrated by Ossowski and Reich for urokinase and the spread of Hep3 cells (1983), tissue plasminogen activator were to have an important role in the dissemination of UCT-Mel 3 derived tumours, it might well be that growth of the tumour to the point where a critical tumour cell:fibroblast ratio was exceeded, would allow tumour spread to occur.

In the fourth place, it may be that tumour cells from UCT-Mel 3 do, indeed, enter the circulation from a very early stage of primary tumour growth but fail to establish themselves as viable metastatic deposits without growth factors (or inhibitors of host responses) that are derived from the primary tumour. Sordat et al., (1978), for example, have shown that nude mice bearing human colorectal carcinomas support the intra-peritoneal survival of the carcinoma cells better than do tumour-free mice, indicating that local tumour growth may have a systemic effect upon distant foci. A certain critical concentration, or rate of delivery, of these factors might be required and this in turn might well be dependent upon the size of the viable primary

tumour. I can think of a number of experiments that may shed light on this possibility. If, for example, a mouse were to develop metastasis after the excision of two tumours, each weighing less than 0.6g but with a combined mass of greater than 1g this would strongly suggest that a humoral factor of some sort was involved in micrometastasis survival. On a slightly different, but in principle similar, tack, it may be possible to inhibit spread of a large UCT-Mel 3 derived tumour by the simultaneous implantation of the non-metastatic variant of UCT-Mel 3 that was derived by repeated in vivo passage and in vitro culture. This would imply that a non-metastatic tumour released inhibitory factors. Experimental support for this possibility comes from the work of Schatten (1958) and Ketcham et al (1961) who have shown that the rate of metastatic spread increased after removal of the primary tumour. Although Gorelike et al., (1978) showed that this phenomenon was frequently tumour-specific, and could therefore involve immunological mechanisms, in the nude mouse these should not apply and this system would thus provide a clearer indication of the mechanisms involved.

The fact of the matter is that I have metastatic and non-metastatic tumorigenic lines at my disposal, and it should be possible to design experimental protocols that would illuminate present areas of uncertainty regarding quantitative relationships between size and metastatic potential.

UCT-Mel 5 proved to be an interesting tumour in several respects. Most prominent among these, perhaps, was the observation that repeated in vivo passage from a metastatic deposit, even though this passage was done by transfer of subcutaneous tumours, gave rise to a tumour variant that spread uniformly. This is the only instance in which I was able to increase the metastatic potential of a line (Table 4.2). Presumably repeated in vivo transfer selected for a clone or subpopulation of cells that possessed an increased innate capacity for invasion and spread, or that was able to circumvent host defences or that was able to interact with the host tissues in such

a way that favoured growth.

Despite the phenotypic drift from low to high metastatic potential, the cells from this line did not lose their organotropic preference for deposition in the lungs. It would be of interest to compare the variant subline with the poorly metastatic parent cells with respect to differences in collagenase production, growth factor requirement, plating efficiency, tendency to aggregate with other blood cells or any of the other many functions that have been associated with acquisition of metastatic behaviour (Fidler et al., 1978). The fact that the aggressive variant line grew more rapidly *in vitro* than the parent line (Table 4.7) is noteworthy.

Repeated *in vivo* transfer did not only lead to an increase in metastatic tendency; in the case of UCT-Mel 2 the cells appeared to lose such potential for spread that they had when first tested. In this case, I presume, the process of repeated *in vivo* transfer selected for a non-metastatic population.

I was unable to detect any correlation between *in vivo* metastasis and *in vitro* cellular release of plasminogen activators, so in this respect my results do not support the belief that this enzyme is an important *constitutive* determinant of spread. It is possible, however, that *in vivo* induction of t-PA activity or the induction of inhibitor synthesis are more important factors than *in vitro* experiments would suggest. The use of antibodies to achieve specific *in vivo* inhibition of t-PA, provide a more realistic indicator of the extent to which this enzyme is involved and the role of plasmin-mediated proteolysis in spread of melanomas in this system should be evaluated after such experiments have been completed.

CHAPTER 5

CHAPTER 5

PHENOTYPIC EVOLUTION OF A HUMAN MELANOMA IN THE NUDE MOUSE

Tumours that develop following the inoculation of malignant cells into an immunological receptive host such as the nude mouse usually grow progressively larger until they reach a stage where they outgrow their blood supply. Necrosis and ulceration then develop, leading to the death of the host if the tumour is allowed to remain *in situ*. The "natural history" of the tumour is usually enacted within the time span of weeks or months - a period that is too short to allow study of interesting phenomena such as progression, dormancy, genetic instability or other forms of phenotypic change that are frequently encountered in human tumours.

By extending the life of the tumour (and hence the period of observation) it has been possible to examine some of these processes. Many strategies have been devised to accomplish this. Ossowski and Reich (1983), for example, transplanted tumours onto the chorio-allantoic membrane (CAM) of the embryonated hen's egg and passaged the tumours that developed on to fresh CAM's or into *in vitro* culture; and Goldenberg and Pavia (1981, 1982) passaged tumours from nude mice into new recipients or re-established the cells in *in vitro* culture. I have shown, in chapter 4, that dormant metastatic deposits will manifest if the life of the host is prolonged by excision of the primary tumour and, in chapter 3, I showed that it is possible to maintain a subtumorigenic inoculum in a dormant state by witholding the estradiol that is required for the tumour to develop. Implantation of an estrogen pellet three weeks after inoculation of the tumour cells then allowed the tumour to grow.

None of these models, however, closely resembles the clinical situation where a tumour is presumed to develop by mutation of a single cell and thereafter to grow at a rate governed by its innate proliferative potential or by genetic influences that regulate dependence on growth factors, blood supply, interaction with host stroma, or other modulators of cell division.

These growth controls, and the responses that they elicit, frequently fluctuate in intensity with the result that the lesion does not increase in size as a monotonic function of time. It is more usual to find that periods of growth alternate with periods of relative dormancy and even regression, culminating eventually, in progression to a more aggressive and rapidly growing phenotype. Progression is though to represent a form of clonal evolution in which an inherently unstable genome (Nowell, 1986) undergoes further changes. The mutants that result are selected for their proliferative advantage so that heterogeneous tumours result with growth characteristics that differ from those of the parent clone.

I was fortunate in that one of the melanoma cell lines (UCT-Mel 7) used for this thesis grew in the nude mouse in a manner that differed from that of tumours derived from other cell lines. Instead of forming, from the start, a relentless and progressive tumour, it showed an initial period of rapid growth followed by a plateau phase, a phase of regression, a phase of dormancy and, finally a phase of rapid exponential growth. All of these phases could be documented in the same host without the need to interfere in any way. In these respects the tumours simulated the growth of spontaneous tumours in man.

In this chapter I present the results of a series of experiments with this tumour that suggest that the phenotypic changes it displayed during the course of its evolution were the results of changes that resulted from host - tumour interactions and were not spontaneous in the random sense.

MATERIAL AND METHODS

The following procedures have previously been described in detail in chapter 1:-

(a) Subcutaneous inoculation of cells into nude mice.

(b) Electrophoretic analysis of lactate dehydrogenase enzymes for

assessment of relative human and murine contribution to tumours of mixed species content.

Collagen content of tumour samples was measured by hydrolysis and assay of the hydroxyproline content of the hydrolysate (Hutterer and Singer, 1960).

RESULTS

As indicated in chapter 1, all of the lines were tumorigenic with the exception of UCT-Mel 6, and tumours that developed from inocula of cells from UCT-Mel 1 through UCT-Mel 5 showed the pattern of relentless growth that is typical of most transplanted tumours. UCT-Mel 7 was a notable exception in this regard. Tumours derived from subcutaneous implants of these cells taken at the 30th *in vitro* passage grew initially at a rapid but decelerating rate so that, within a matter of weeks after appearance of the tumour, it reached a plateau. This was in striking contrast to the continued growth that developed from the other cell lines, of which UCT-Mel 3 provides the example presented in Fig. 1.

Having observed this growth pattern with 30th passage cells, I recovered the 10th passage cells from liquid nitrogen storage to see if they would show a similar pattern. As shown in Fig. 2, these cells grew exponentially in the nude mouse and did not show the "plateau" effect; cells from the 26-30th passage behaved as did those from the 30th passage and showed an initial phase of growth followed by a plateau. Cells from the 52nd passage *in vitro* gave rise to tumours that grew so slowly that it was not possible to discern a phasic growth pattern. They showed, rather, a period of prolonged dormancy with eventual exponential growth. When mice bearing tumours derived from *in vitro* cultures of the 26-30th passage of UCT-Mel 7 cells were observed for longer periods of time, the interesting and consistent growth pattern seen in Fig. 3 was noted. The growth and plateau phases described above were still

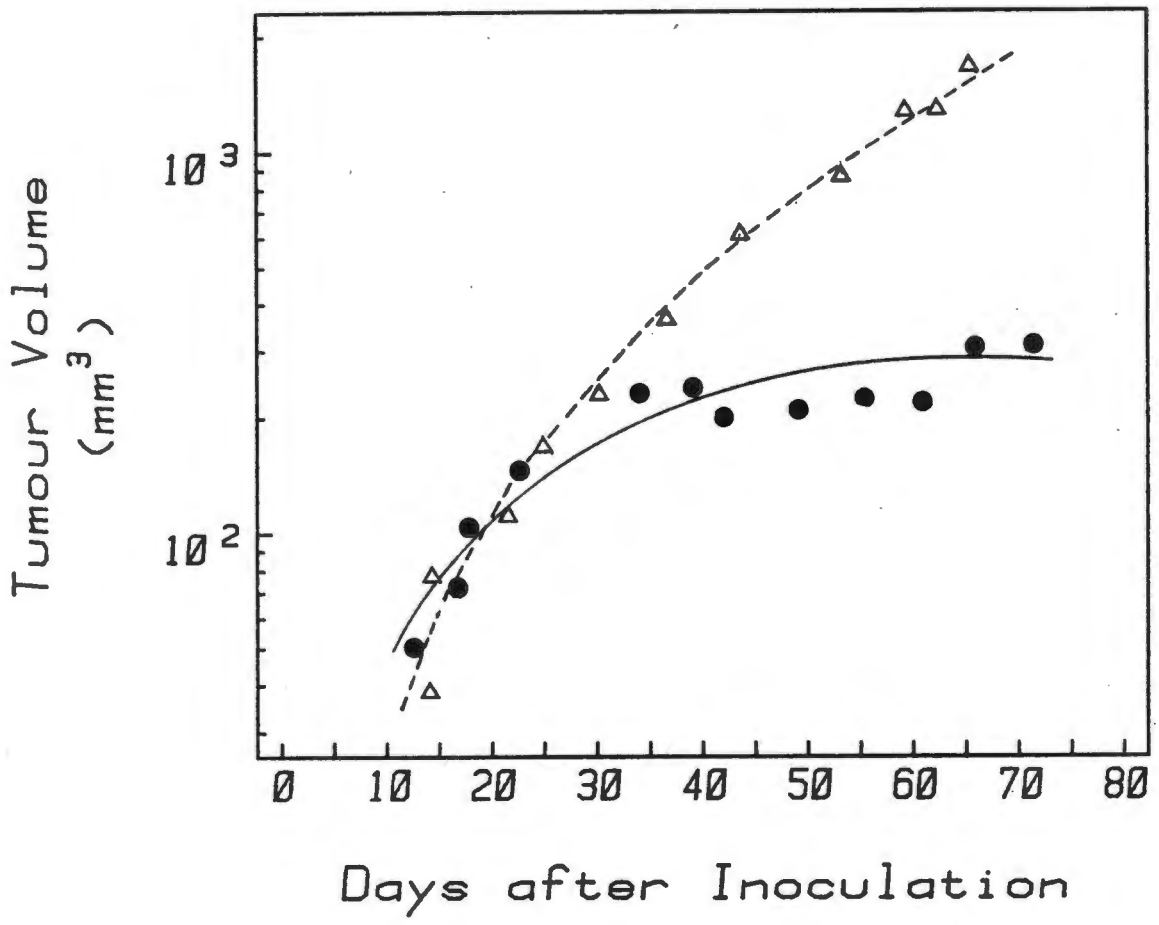


Figure 1

Growth of UCT-Mel 7 and UCT-Mel 3 in nude mice

Mice were inoculated subcutaneously with 10^6 UCT-Mel 7 (●——●) (in vitro passage number 30') or 10^6 UCT-Mel 3 (△----△) (in vitro passage number 69') on day 0 and tumour volumes were measured at the indicated times. The lines on the graph were calculated using the Gompertz function and they represent the mean values for 5 animals.

Note that UCT-Mel 7 grew initially at a rapid but decelerating rate (30 days) after which tumour growth ceased. This was in contrast to the continued growth pattern observed in UCT-Mel 3.

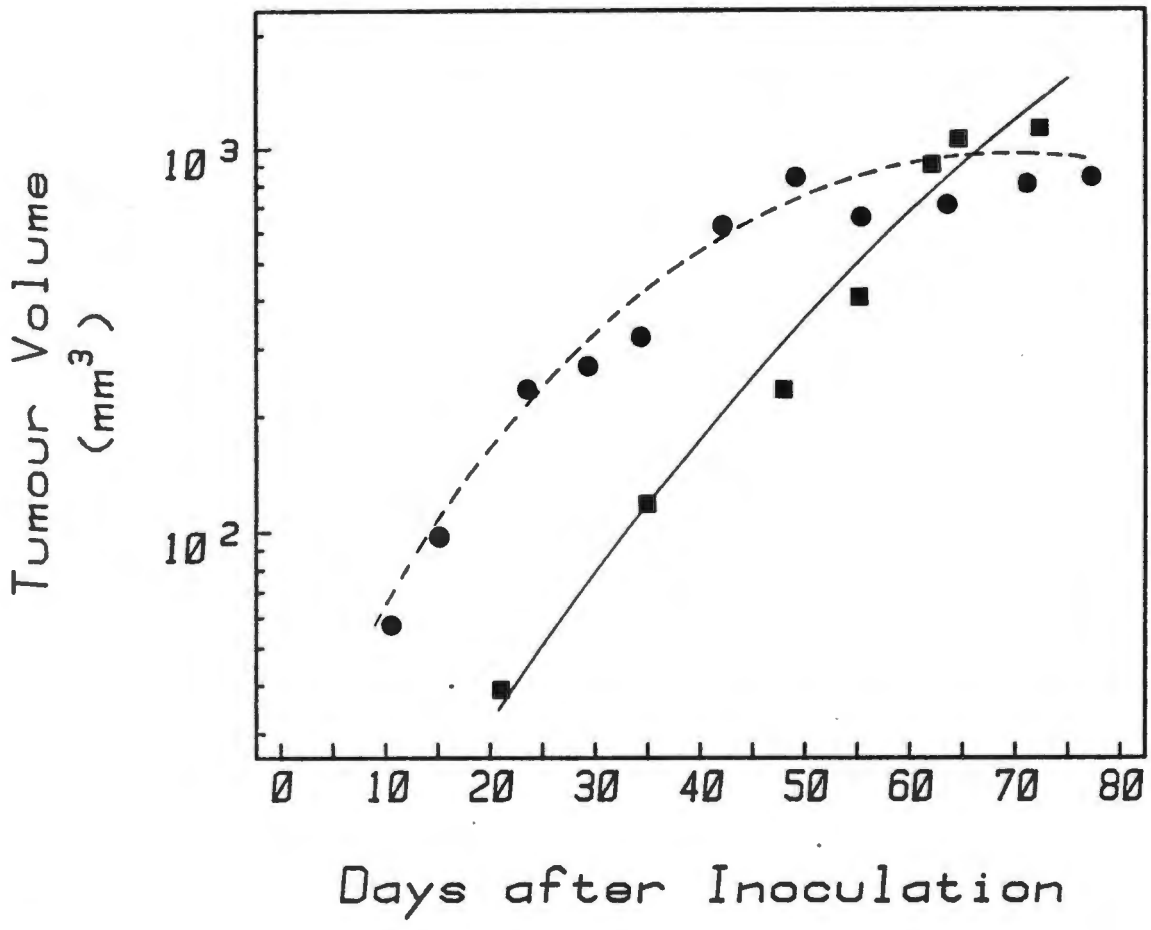


Figure 2

The in vivo growth patterns of UCT-Mel 7 tumours derived from passage 10' and passage 30' in vitro cultured cells

Mice were inoculated subcutaneously with 5×10^6 UCT-Mel 7 cells of in vitro passage number 10' (■——■) or passage number 30' (●----●) on day 0 and tumour volumes were measured at the indicated times. The lines on the graph were calculated using the Gompertz function and they represented the mean values for 5 animals.

Note that UCT-Mel 7 cells from an early passage (10') in vitro grew exponentially in the nude mice and did not show the "plateau" effect exhibited by cells of a later passage (30').

FIGURE 3

Figure 3

The Phasic growth pattern of UCT-Mel 7 tumours in nude mice

Mice were inoculated subcutaneously with 5×10^6 UCT-Mel 7 cells of in vitro passage number 30' (Figs. 3a and b) on day 0 and tumour volumes were measured at the indicated times. Lines in the figures were constructed by plotting tumour volumes as a function of time for a single mouse.

Note that the initial growth phase (1) was followed by a plateau phase (2) during which no increase in size of tumours was observed. This period was followed by a phase of tumour regression (3). This regression phase was usually followed by a period of dormancy (4) after which an exponential growth phase was observed (5)

Fig. 3(a) demonstrates these phases of growth in a single mouse tumour.

Fig. 3(b) shows a variation which was occasionally observed. The exponential phase (5) followed the regression period (3) without a preceding dormant phase (4).

Figure 3a

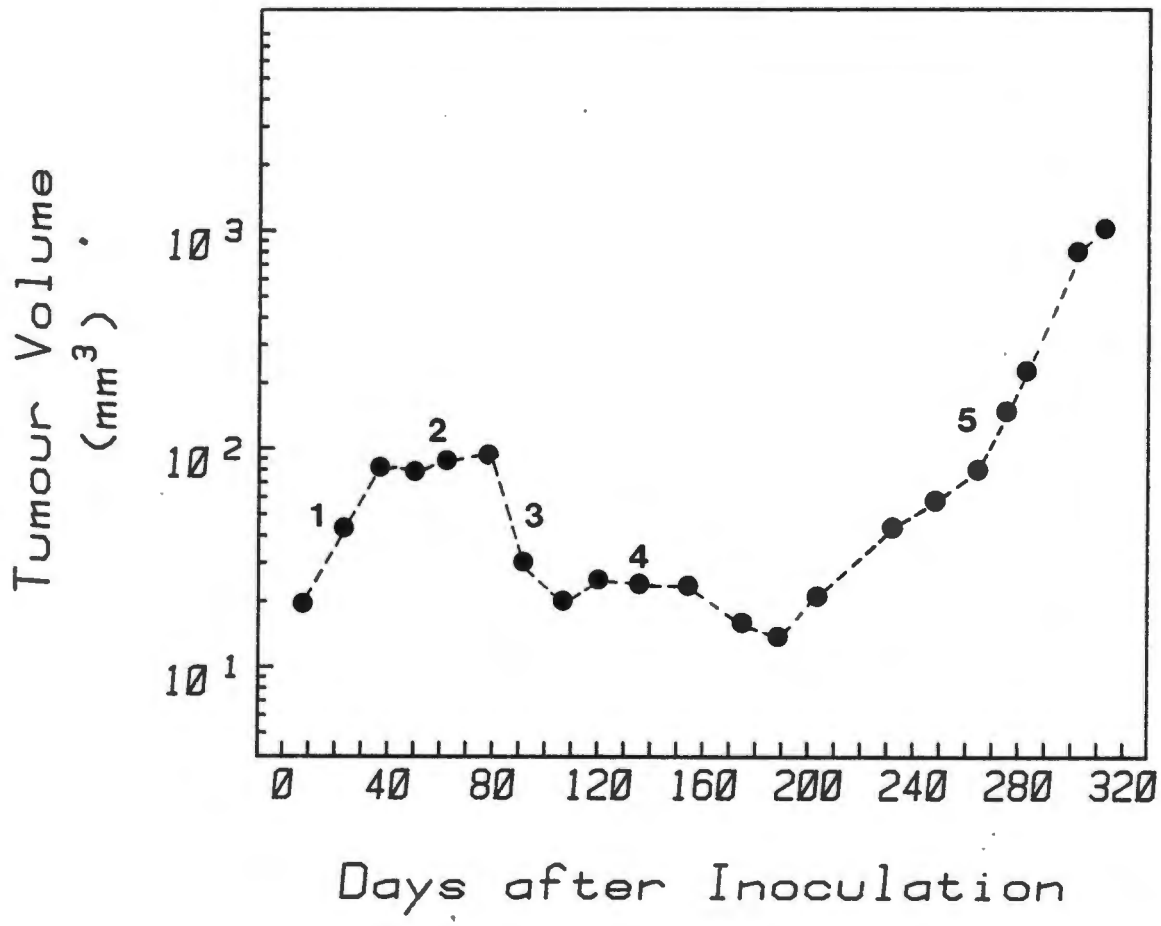
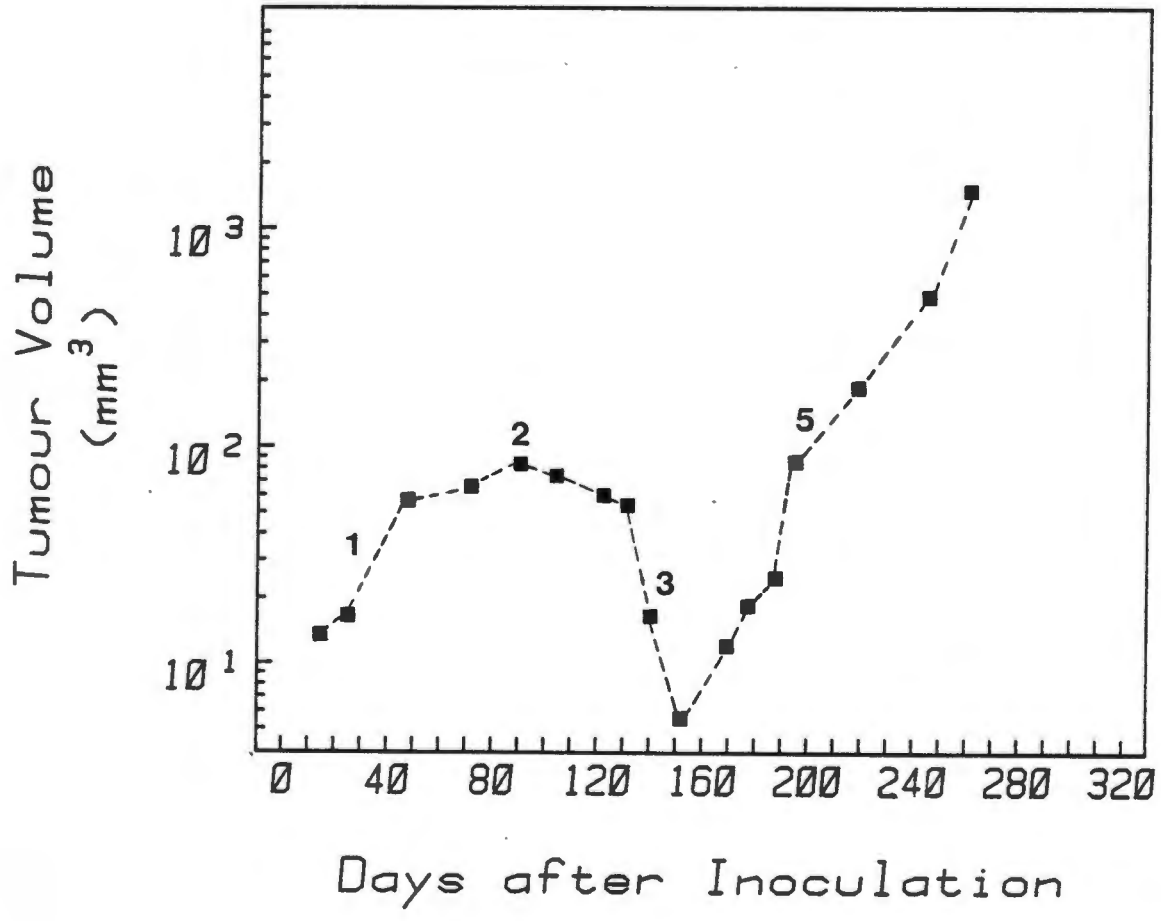


Figure 3b



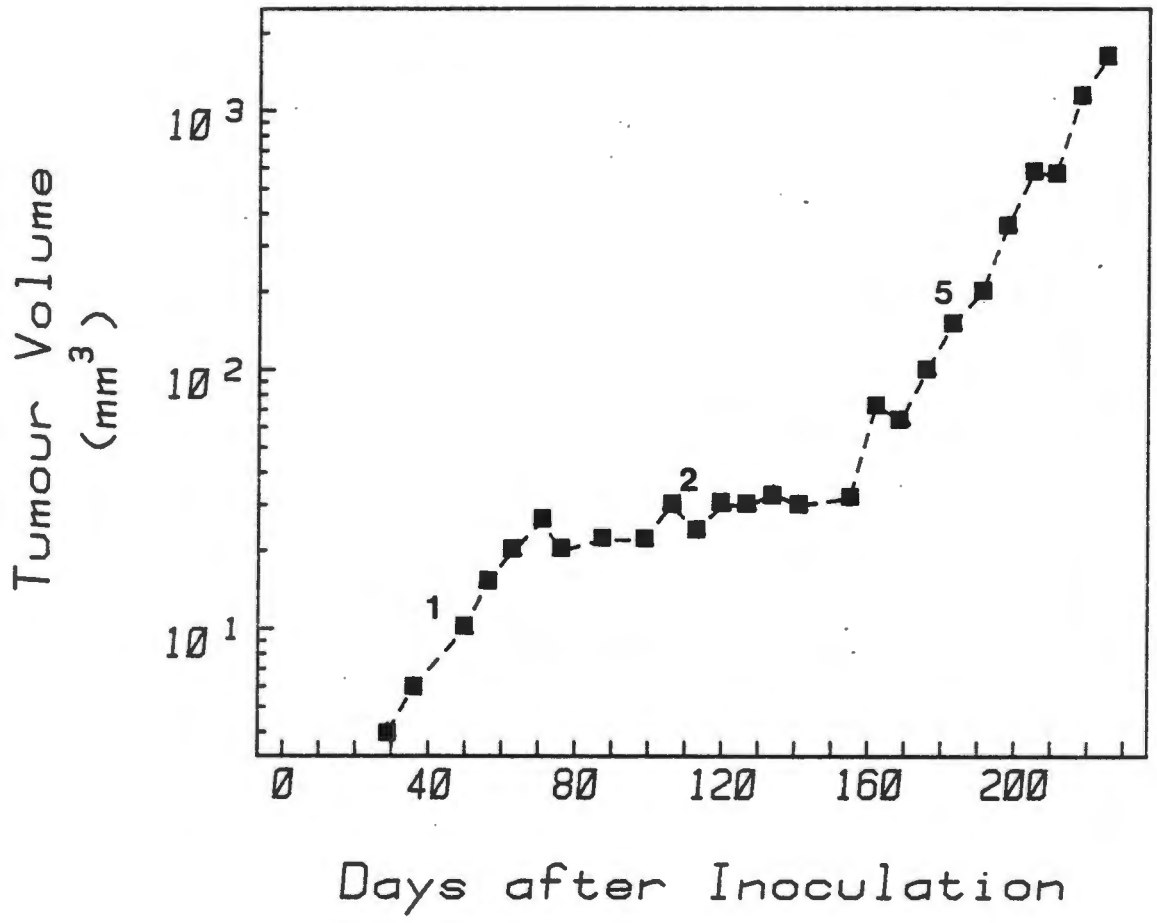


Figure 3c

The phasic growth pattern of UCT-Mel 7 tumour in nude mice

A mouse was inoculated subcutaneously with 10^6 UCT-Mel 7 cells of in vitro passage number 56' on day 0 and tumour volumes were measured at the indicated times. The line in the figure was constructed by plotting tumour volumes as a function of time.

Note that the initial growth phase (a) was followed by a plateau phase (2) during which no increase in size of the tumour was observed. This period was followed by a phase of exponential growth (5), without a preceding regression and dormant phase (3 and 4).

observed but these were followed by regression to a small, barely palpable nodule, a variable period of dormancy and, finally, a sudden escape from latency to give rise to an extremely rapidly, exponentially growing tumour.

I refer to this as the "phasic" growth pattern since five phases could readily be discerned:

(1) A growth phase;

(2) A plateau phase;

(3) A phase of regression;

(4) A period of dormancy; and, finally

(5) A period of exponential growth. (Fig. 3).

In table 1 I summarise the data from 3 experiments in which the time relationships of these five stages were measured, together with the doubling times of the tumours as they grew during the exponential phase. It is evident that, once this stage had been reached, the tumours all grew with the same *in vivo* doubling times.

Tumours that were removed during the phase of exponential growth and reimplanted into fresh recipients formed tumours (which I refer to as 5P) which also grew exponentially. (Fig. 4). These showed no tendency to revert to the phasic growth pattern. During the course of its development in the donor mouse, therefore, the tumour changed its phenotype completely to that of an aggressively growing tumour.

As indicated above, cells taken at the 10th *in vitro* passage grew exponentially. (Fig. 2). When a fragment of such an exponentially growing tumour was removed and re-implanted, the phasic growth phenotype was seen. (Fig. 5).

It was of interest to observe that there was a paradoxical inverse relationship between the *in vivo* and *in vitro* proliferative rates of the two tumour phenotypes. An exponentially growing tumour, for example, with an

Table 1

Mouse No.	No. of cells inoculated ($\times 10^{-6}$)	Phase Duration (days) 1	2 (volume cu.mm)	3	4	(a) Total (days)	Phase 5 (days) (b) T:2
1	1	50	98 (30)	*	*	148	12
2	1	48	43 (50)	*	*	91	23
3	1	82	86 (112)	*	*	168	42
4	5	56	42 (90)	22	21	141	11
5	5	34	51 (143)	29	88	202	16
6	5	26	65 (60)	23	104	218	14
7	5	42	49 (85)	69	84	238	12
8	5	70	(c)	65	62	197	9
9	5	42	90 (70)	22	(d)	154	24
10	5	35	27 (138)	14	77	153	12

(a) This column represents the total time to the onset of phase 5.

(b) T:2. Tumours grew exponentially during phase 5 and the figure in this column represents the time taken for the tumours to double in volume.

* Mice inoculated with 10^6 cells did not show a regression phase followed by a dormant phase. Their dormant phase (4) and plateau phase (2) coincided.

(c) In this instance regression (Phase 3) followed the growth phase (Phase 1)

(d) In this instance growth commenced immediately after the regression phase (Phase 3).

FIGURE 4

Figure 4

Growth patterns of tumours derived from in vitro or in vivo passages of UCT-Mel 7

(a) Mice were inoculated subcutaneously with either 5×10^6 UCT-Mel 7 cells of in vitro passage number 29' (●----●) or with 10^6 UCT-Mel 7 cells which had been derived from exponentially growing tumours passed 10 times through nude mice and passaged for 10 passages in vitro (■-----■). Cells were inoculated on day 0 and tumour volumes were measured at the indicated times. The lines on the graph were calculated using the Gompertz function and they represent the mean values for 5 animals.

Note that tumours that arose from cells that had been passaged through nude mice and then in vitro grew exponentially. Those that arose from cells that had been passaged exlusively in vitro showed the phasic growth pattern.

(b) Mice were inocualted subcutaneously with either 10^6 UCT-Mel 7 cells of in vitro passage number 52' (○——○) or with 10^6 UCT-Mel 7 cells which had been derived from exponentially growing tumours passed 8 times through nude mice and passaged for 10 passages in vitro (▼——▼). Five mice were inoculated in each case.

Note that tumours that arose from cells that had been passaged through nude mice and then in vitro grew exponentially. Those that arose from cells that had been passaged exclusively in vitro showed the phasic growth pattern.

Figure 4a

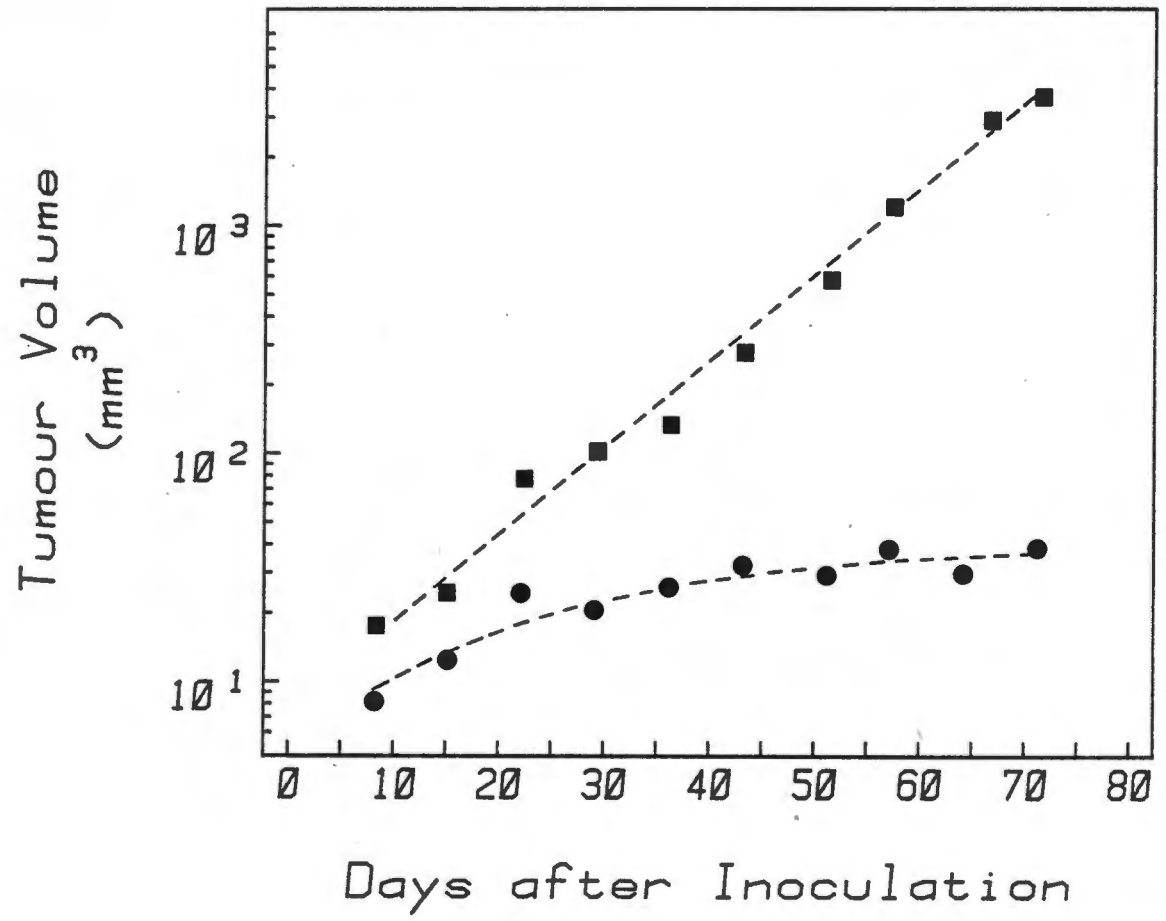
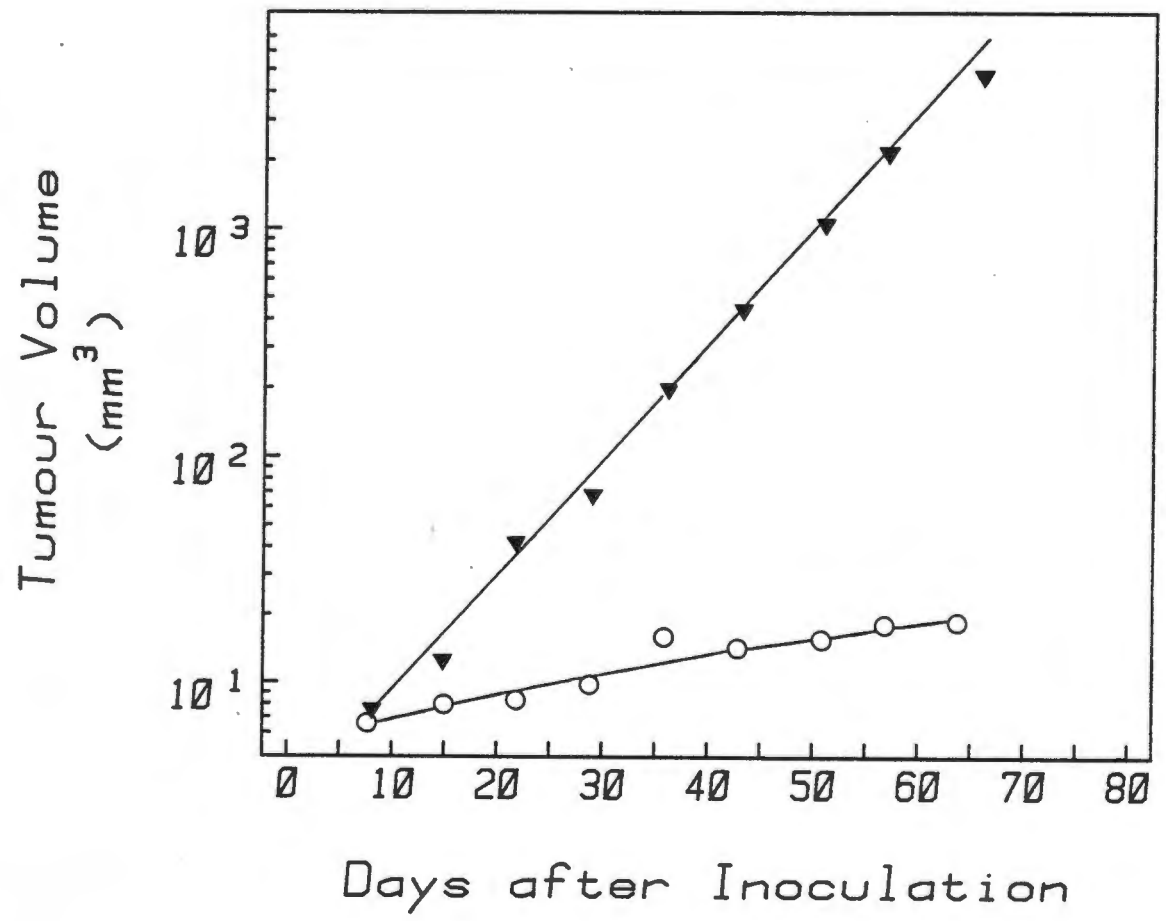


Figure 4b



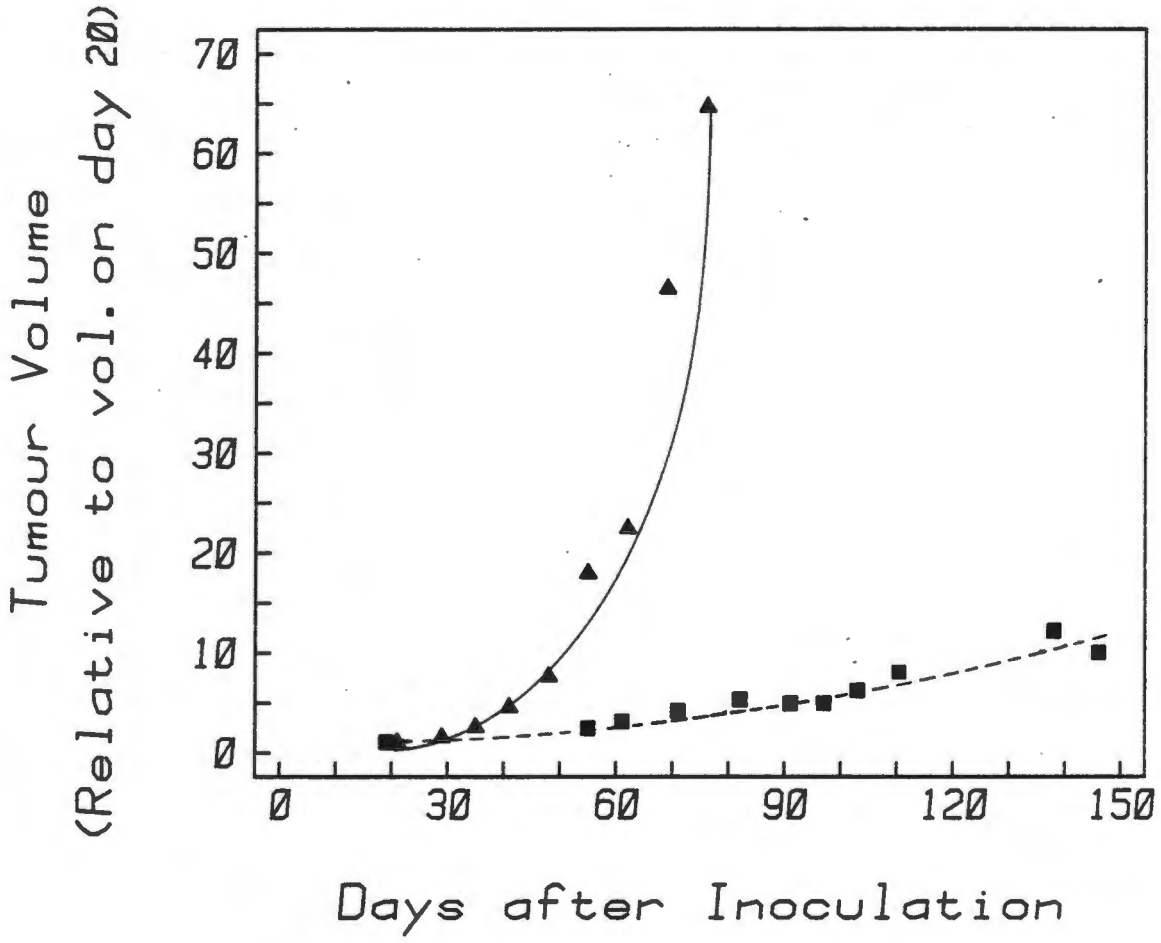


Figure 5

Mice were inoculated subcutaneously with 10^6 UCT-Mel 7 cells (in vitro passage number 8') (▲——▲) or implanted subcutaneously with fragments obtained from one of these tumours (■- - -■). Cells or tumour fragments were introduced on day 0 and tumour volumes were measured at the indicated times. The tumour volumes are expressed relative to the volumes of both sets of tumours scored on day 20.

Note that tumours that arose from 8' cells grew exponentially. Tumours that arose from fragments removed during this exponential growth displayed an altered growth pattern and grew exceedingly slowly with an in vivo doubling time of 30 days compared with 12 days for the exponentially growing tumours (▲——▲).

in vivo doubling time of 8.0 days had an in vitro doubling time of 141 hours. The tumours showing the phasic growth pattern were derived from cells with a much shorter doubling time of 47 hours. (Fig. 6).

The histological appearances of the tumours removed at different stages of their in vivo evolution from phase 2 to phase 5 phenotypes differed considerably. As can be seen from the photomicrographs reproduced in Fig. 7 tumours removed during phase 1 or phase 2 were remarkable for their differentiated appearance and for their extensive network of reticulin fibre formation. (Fig.7a and b). Tumours that were removed during phase 5 were less obviously differentiated and reticulin deposition was far less pronounced. (Fig. 7c) Phase 5P tumours (i.e. those removed during the exponential phase from one mouse and transplanted into a new host), also grew exponentially and showed scant reticulin fibre formation. Like the phase 5 tumours from which they were derived, they too showed a poorly differentiated morphology. Phase contrast microscopy of in vitro cultures revealed notable differences between cells that gave rise to tumours with the phasic growth pattern and cell lines derived from tumours removed during phase 5 or phase 5P.

Between the 10th and the 53rd in vitro passage cells retained their predominantly elongated, spindle shaped morphology (Fig. 8a-1;a-2;a-3), but differed in their in vitro rates of proliferation (95 hours at 10'; 47 hours at 53' and in the maxiumal cell density they reached. Confluent late passage cultures were very much more dense than earlier passages.

Fragments of phase 5 tumours were serially transplanted into fresh recipient hosts and then returned to in vitro culture. Such cultures differed to a greater of lesser extent with each other and with the original parent line in terms of their appearance under phase microscopy. They were consistently similar, however, in that they proliferated very slowly in vitro and, paradoxically, formed rapidly growing phase 5P tumours when re-inoculated into nude mice.

FIGURES 6a and 6b

Figure 6

Relationship between growth patterns of UCT-Mel 7 in vitro and in vivo

(a) UCT-Mel 7 cells at the 52' passage recovered from liquid nitrogen storage (▲——▲) or fragments from a tumour removed during the exponential growth phase and passaged 10 times through mice and established in culture for 10 passages (●——●) were seeded in RPMI-FC10 on 35 mm dishes on day 0. The medium was changed 24 hr later and thereafter at 48 hr intervals. At the indicated times, replicate cultures were trypsinized and the cells counted in a Coulter counter.

(b) 10^6 UCT-Mel 7 cells at the 52' passage in vitro (▲——▲) or 10^6 UCT-Mel 7 cells derived from an exponentially growing tumour passaged for 10 passages through nude mice and established in culture for 10 passages (●——●) were inoculated subcutaneously into nude mice, on day 0 and tumour volumes were measured at the indicated times. The lines in the figure were calculated using the Gompertz function and they represent the mean values for 5 animals.

Note that an exponentially growing tumour (Fig. 6b, ●——●), with an in vivo doubling time of 8.0 days was obtained from the inoculation of cells with an in vitro doubling time of 141 hrs (Fig. 6a, ●——●), whereas the tumours showing the phasic growth pattern (Fig. 6b, ▲——▲) were derived from cells (52') with a much shorter doubling time of 47 hours (Fig. 6a, ▲——▲).

Figure 6a

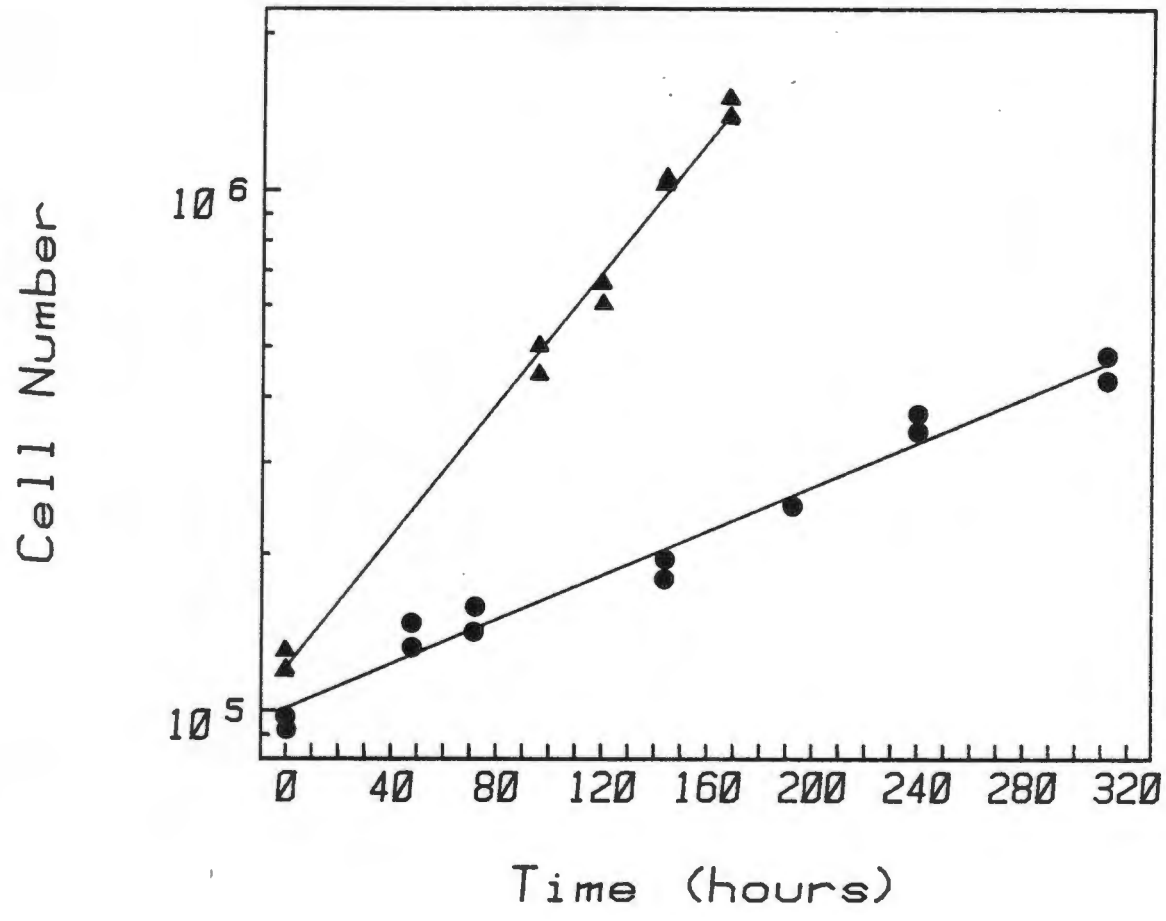


Figure 6b

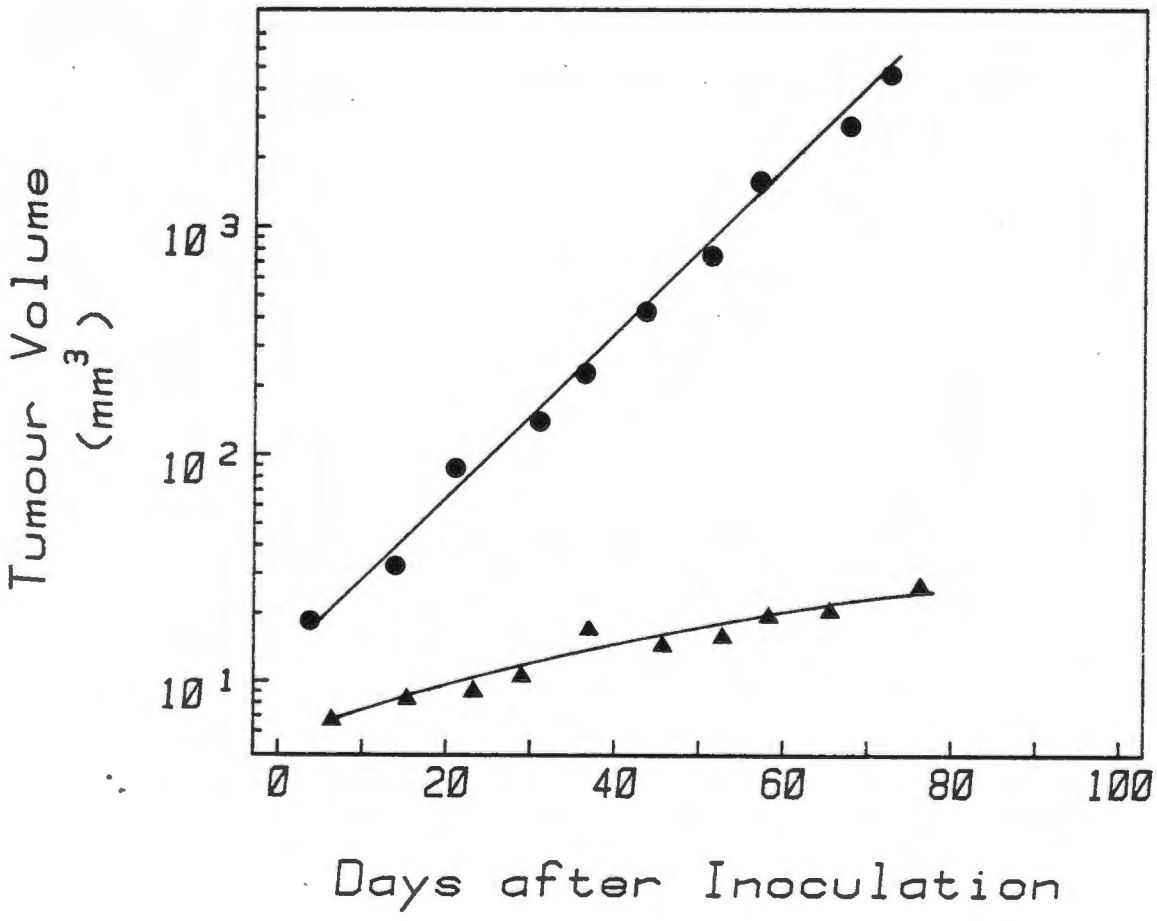


FIGURE 7

Figure 7

Morphology of UCT-Mel 7 tumours removed during different phases of growth.

The composite figure portrays photomicrographs of the histological appearance of UCT-Mel 7 tumours removed at different stages of their in vivo evolution from phase 1 to phase 5P. The left half of each photograph shows tumours stained by the H&E method; the right half of each photograph shows the same tumours stained for reticulin (Gordon and Sweet, 1936).

(a) These photographs indicate the appearance of tumours removed during phase 1. They portray a spindle cell malignant melanoma with a herring bone fasciculated pattern containing fairly well formed blood vessels. Abundant reticulin fibre formation surrounding individual cells is evident. These attributes are those of a well differentiated tumour.

(b) These photographs indicate the appearance of tumours removed during phase 2. They also show a tumour composed of spindle shaped melanoma cells but the herring bone pattern noted in (a) is less obvious. Reticulin fibre formation is also abundant and is present in a swirling pattern which usually surrounds individualy tumour cells. This pattern also indicates a well differentiated tumour.

(c) These photographs indicate the appearance of tumours removed during phase 5 i.e. the exponential phase of growth. Striking differences between (a) and (b) can be noted. The tumour has a far less differentiated appearance and the herring bone pattern is no longer detectable. The tumour is still composed of spindle shaped cells. Reticulin fibre formation is virtually absent.

(d) These photographs indicate the appearance of tumours removed during phase 5P (i.e. exponentially growing tumours passaged through new animals). The tumours show a spindle cell malignant melanoma which has a barely detectable swirling pattern. More reticulin than that present in (c) is observed. The reticulin can be noted to be concentrated around supporting structures such as vessels. Much less reticulin is evident that is apparent in (a) and (b) and the morphological pattern of these tumours is indicative of a less well differentiated tumour. The scale marker in (d) represents 100 μm. All photographs are at the same magnification.

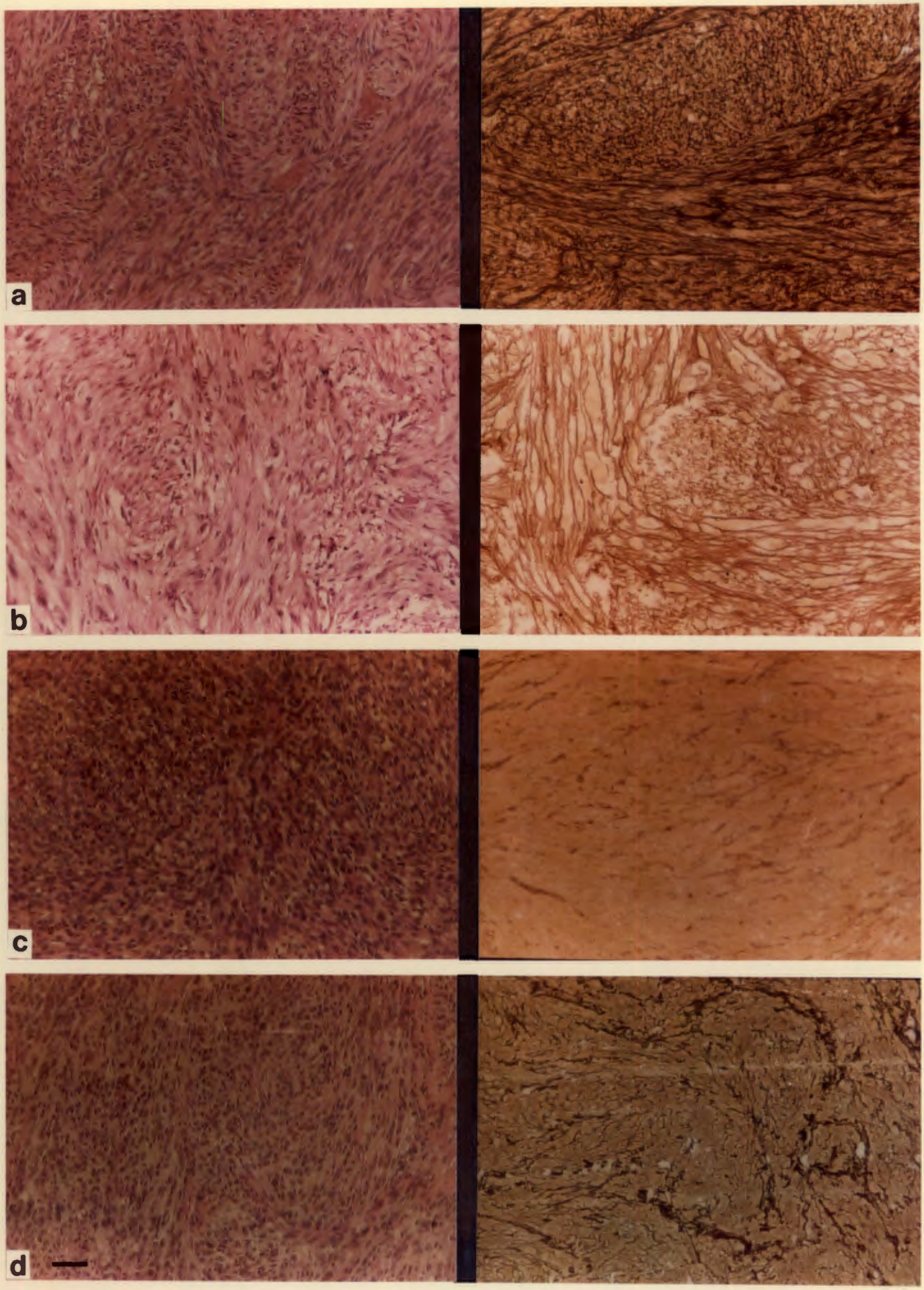


Figure 7

FIGURE 8a

Figure 8a

Phase contrast photographs of UCT-Mel 7 cells

Fig. 8a-1. Parent UCT-Mel 7 cells of passage 10' in vitro. Note the spindle shaped cells with pronounced cytoplasmic processes. Cells grew to a low density.

Fig. 8a-2. Parent UCT-Mel 7 cells of passage 29' in vitro. The cells still had a spindle morphology with cytoplasmic extensions. Cells had a tendency to grow in chords.

Fig. 8a-3. Parent UCT-Mel 7 cells of passage 53' in vitro. In sparse cultures a spindle morphology was still evident. This photograph shows that the cells from this passage grew to a far higher density than those from earlier passages. They also had an increased growth rate (see text).

Scale marker in (3) represents 50 μm.

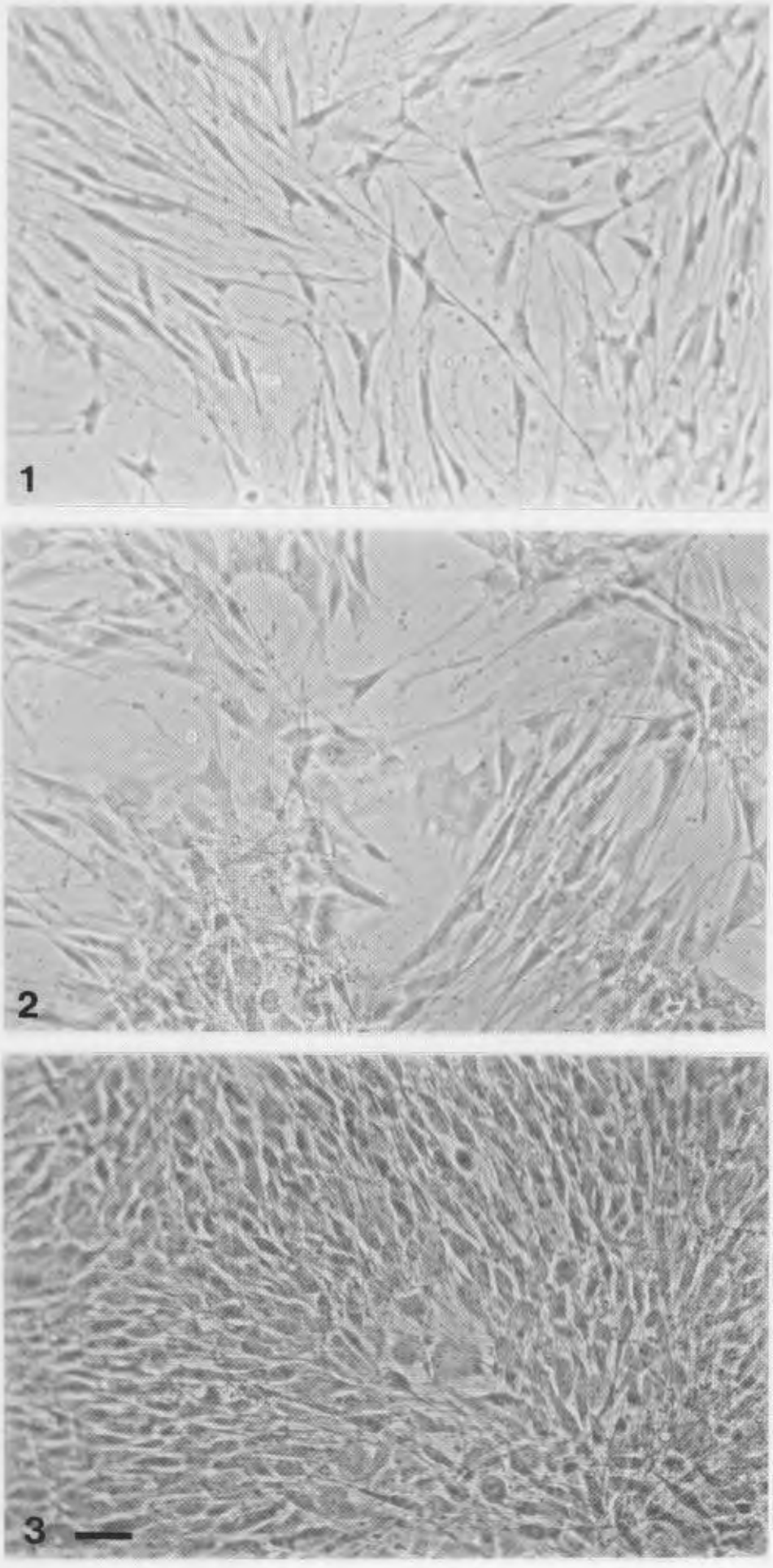


Figure 8a

Figs. 8b.1 and 8b.2 are representative of two such *in vitro* cultures. The cells shown in Fig. 8b.1 had a history of having been passaged 30 times *in vitro*, serially transplanted 8 times in nude mice (in all cases as exponentially growing phase 5P tumours) and then passaged a further 8 times *in vitro*. These cells had a doubling time in culture of 282 hours and a squat, small-celled appearance.

Fig. 8b.2 shows the spindle-celled appearance of a culture that was first inocuated into mice at the 30' *in vitro* passage. The phase 5 tumour that resulted was serially transplanted 10 times in mice as a rapidly growing phase 5P tumour, and it was finally repeatedly passaged in culture. The microphotograph shows the cells after the 10th such passage. At this stage the cells closely resembled, in their morphology, the original cells from which they were derived. They differed greatly, however, in the rates at which they proliferated *in vitro*. These cells had a doubling time of 141 hours; the original line (at the 30th passage) had a doubling rate of 52 hours.

On a number of occasions the tumours that grew during phase 5 were deeply pigmented and, on histological examination, intense melanin deposition was readily seen (Fig. 9a and b). Phase 1 tumours were invariably amelanotic. Despite this apparent differentiation from the amelanotic to the pigmented pheotype, phase 5 tumours that were pigmented grew at the same rate *in vivo* as their amelanotic counterparts. (Fig. 10). The *in vitro* cell lines which were established from phase 5 melanotic tumour samples were always amelanotic.

Tumours removed during the phase 1 and those excised during phase 5 differed strikingly in two other respects.

Firstly, phase 1 tumours were heavily infiltrated with murine fibroblasts and collagen whereas exponentially growing neoplasms showed virtually no such reticulin (Fig. 7). These histological appearances were confirmed by chemical estimation of the hydroxyproline content of tumours removed during phases 2 and

FIGURE 8b

Figure 8b

Phase contrast photographs of UCT-Mel 7 cells

Fig. 8b-1 This photograph shows a cell line derived from UCT-Mel 7 tumours passaged during their exponential phase 8 times through mice. The cells had been passaged through 9 in vitro passages. The cells are small and squat and differ markedly in appearance from the parent line. They grew extremely slowly in vitro (282 hr doubling time).

Fig. 8b-2 This photograph shows a cell line derived from UCT-Mel 7 tumours passaged during their exponential phase 10 times through mice. The cells had been passaged through 10 in vitro passages. The cells are spindle shaped with long cytoplasmic processes and resemble the original parent cells morphologically. They, also however, grow extremely slowly in vitro (141 hr doubling time).

Both these cell lines are of human origin.

Scale bar in (b) represents 50 μm.

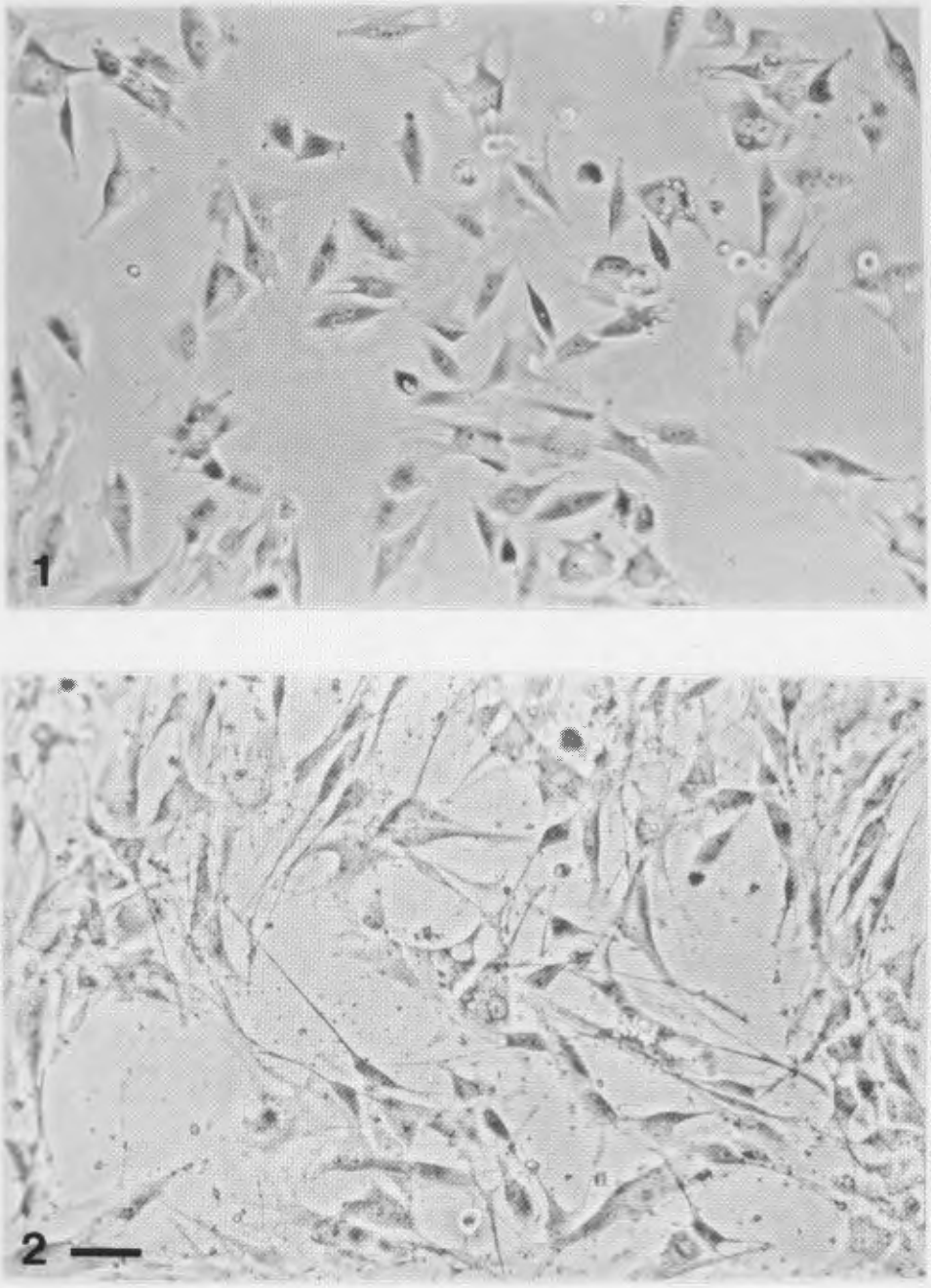


Figure 8b

FIGURES 9a and 9b

Figure 9a

The appearance of UCT-Mel 7 tumours on removal from nude mice

The gross appearance of UCT-Mel 7 tumours removed from nude mice during phase 5P, varied from non pigmented to deeply pigmented. This figure shows 2 such representative tumours.

Each small division corresponds to 1 mm.

Figure 9b

The histological appearance of a pigmented UCT-Mel 7 tumour removed during phase 5P in the nude mice.

This section shows malignant melanoma cells organized in a herring bone pattern. Spindle shaped and epitheloid cells are present. Intracellular melanin can be noted in some of the cells.

Melanin deposits were revealed by the method of Masson.

Scale marker corresponds to 20 μm.

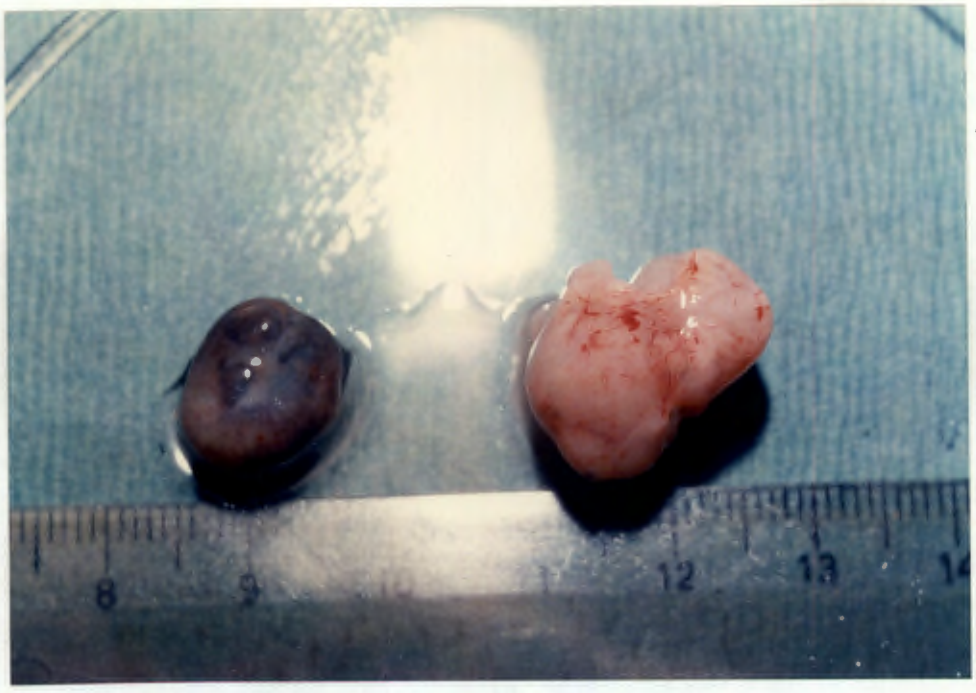


Figure 9a

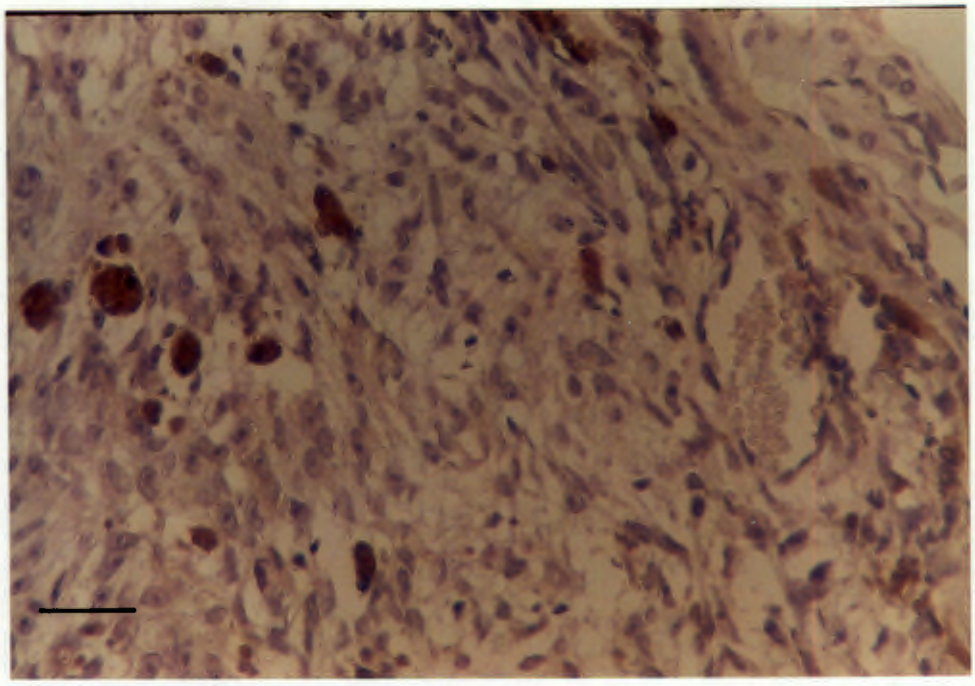


Figure 9b

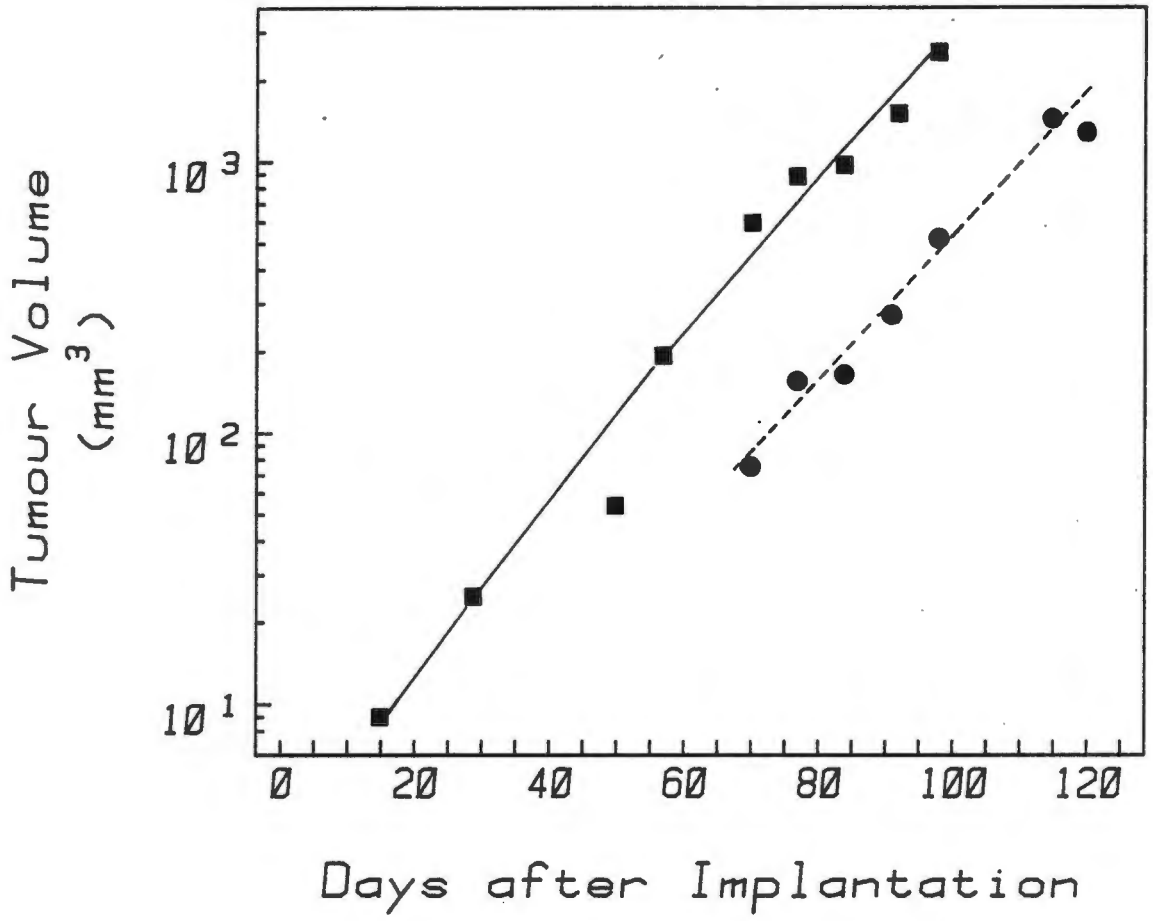


Figure 10

Growth in vivo of pigmented and non-pigmented UCT-Mel 7 tumours

A pigmented (■——■) and an amelanotic (●----●) melanoma were each removed from different mice. Fragments from these tumours were implanted subcutaneously into new recipients and tumour volumes were measured at the indicated times. Similar doubling times in vivo (9.6 and 10.3 days respectively) were recorded.

5 and removed during the subsequent passages (phase 5P) of these tumours in mice. (Fig. 11).

Secondly, early passages of cultures established from tumours that had been removed during phase 2 showed extensive infiltration with murine macrophages and fibroblasts. (Fig. 12a and b). This appearance was not observed to the same extent when phase 5 tumours were examined with this technique.

Infiltration of the tumour with murine mesenchymal and stromal elements could readily be documented by electrophoretic analysis of lactic dehydrogenase enzymes that were present. (Fig. 13). In most cases the human enzyme predominated although varying amounts of the murine species were usually observed. (Table 2).

On two surprising occasions, tumours removed during phase 5 and passaged serially gave rise to cell lines which contained exclusively the murine enzyme with no trace of a human band. (Fig. 14). Karyotypic analysis of these tumours showed exclusively mouse chromosomes and fragments of the tumours grew exponentially when transplanted into immunocompetent nu/+ heterozygous mice. I feel certain, therefore, that the tumours that developed in these two instances were murine and not human. It should be stressed that the tumours that did develop arose in the site previously occupied by the small "dormant" phase 3 nodule.

Table 2 summarises the results of lactate dehydrogenase analysis on five phase 5 tumours which were removed and passaged serially through mice. At the indicated passage numbers fragments of tumour were removed for <u>in vitro</u> culture and cell lines were established which were also examined for lactic dehydrogenase content. As can be noted two of the established cell lines were of human origin, one still contained murine cells and in two instances the enzyme was that of mouse origin.

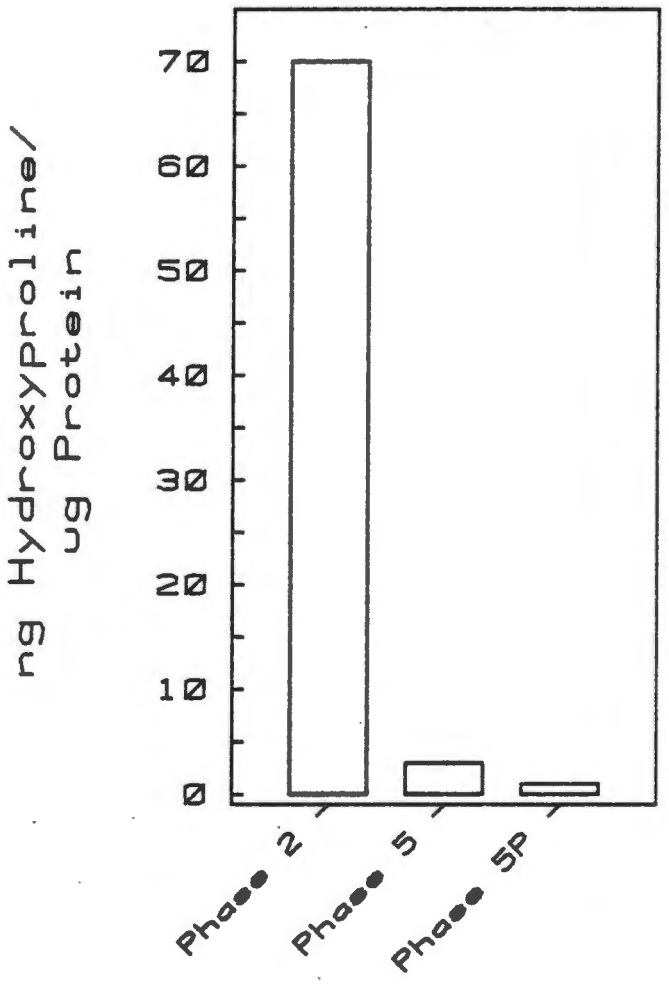


Figure 11

Collagen content of UCT-Mel 7 tumours removed at different phases of growth

The collagen content (measured as ng of hydroxyproline per ug of cellular protein) of UCT-Mel 7 tumours removed at different phases of their growth was determined. The figure is constructed from the results of 6 phase 2 tumours, 4 phase 5 tumours and 12 phase 5P tumours. (Individual data are presented in the Appendix Table A.16).

Note that phase 2 tumours (i.e. the plateau period) evoked an intense desmoplastic response.

FIGURE 12

Figure 12

Phase contrast photographs of UCT-Mel 7 cells showing contamination with mouse macrophages and fibroblasts

Fig. 12a A UCT-Mel 7 tumour was removed during phase 2 and placed in culture. A nest of tumour cells can be seen to be surrounded by fibroblasts. Host macrophages (stellate and round cells) are present in abundance.

Fig. 12b A UCT-Mel 7 tumour was removed during phase 2 and placed in culture. Tumour cells can be noted in the centre of the picture and are surrounded by mouse fibroblasts. Macrophages are not as abundant as in Fig. 12(a).

The scale marker in (b) represents 50 μm.

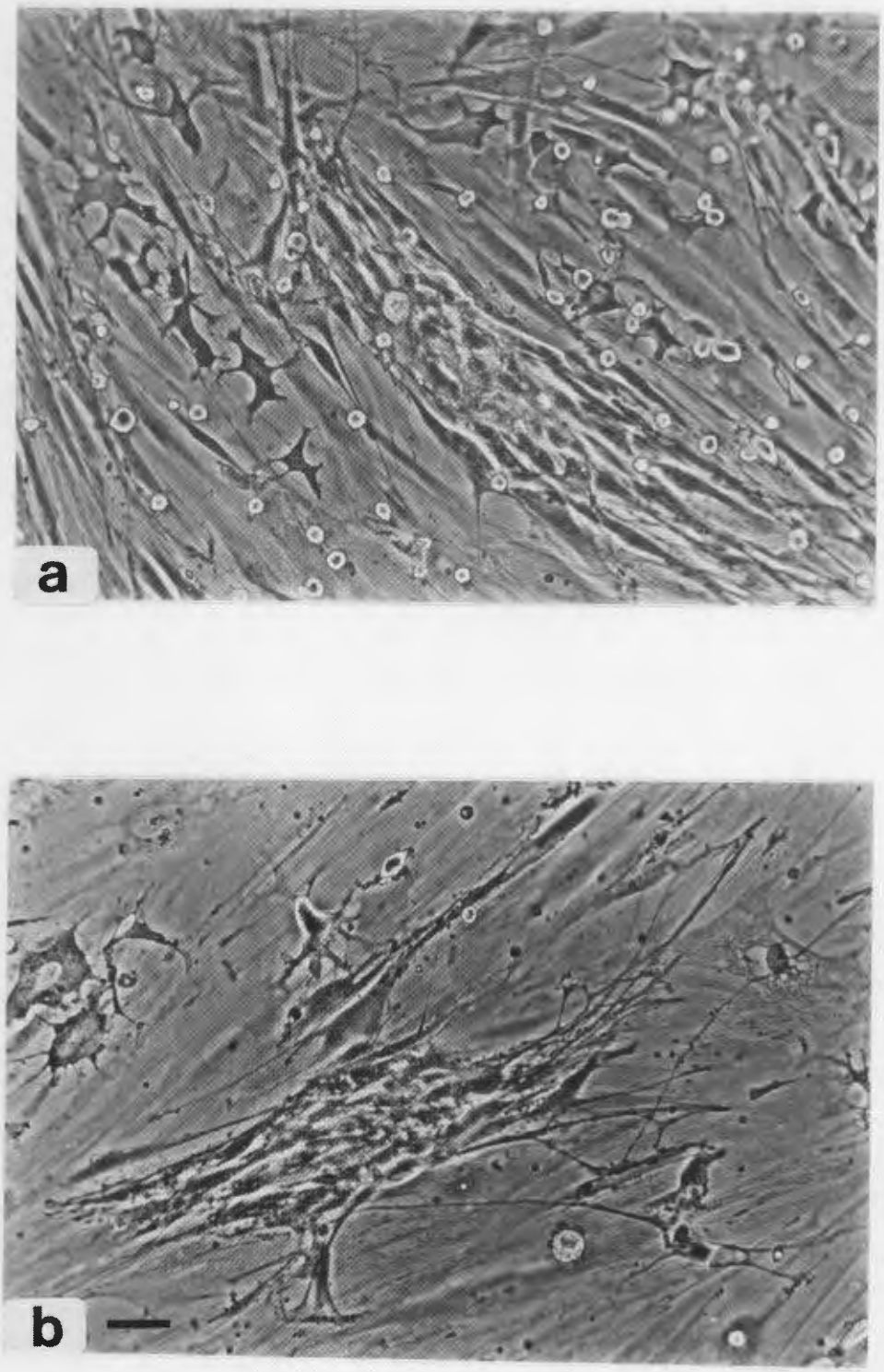


Figure 12

FIGURE 13

Figure 13

Lactate dehydrogenase analysis on human tumours removed from nude mice

Mice were inoculated subcutaneously with either 10^6 or $5x10^6$ UCT-Mel 7 cells (in vitro passage numbers 26', 29', 30'). These tumours grew exponentially for a period of approximately 80 days after which growth ceased and tumours regressed. Four to 7 months after initial inoculation of these tumour cells tumours started growing once more. They now grew vigorously showing exponential growth kinetics. At this stage tumours were removed and small explants were implanted subcutaneously into new animals. The tumours were homogenized and LDH activities determined.

Samples of tumour homogenates were as follows:-

Channel 1, 2 and 3 - UCT-Mel 7 tumour tissue homogenate from passage 6, 7and 7 respectively, showing LDH enzyme bands of both human and mouse origin.

Channel 4 - mouse liver homogenate, showing LDH enzyme band of mouse origin.

Channel 5 - human breast homogenate, showing LDH enzyme band of human origin.

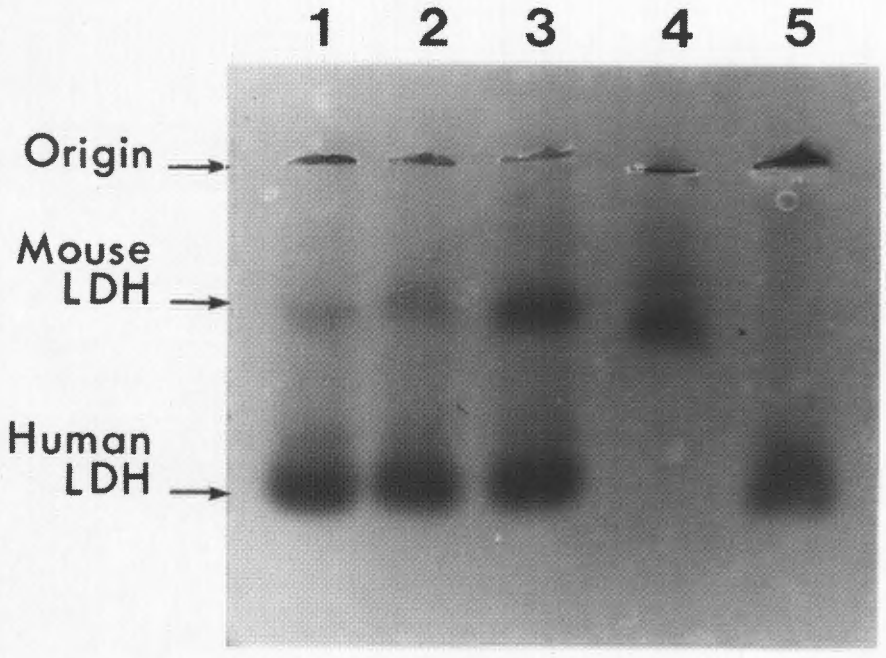


Figure 13

FIGURE 14

Figure 14

Lactate dehydrogenase analysis on UCT-Mel 7 cells originating from tumours removed during phases 5P growth in vivo.

UCT-Mel 7 tumours removed during phase 5P and serially passaged in vitro gave rise to cell lines which contained exclusively the murine enzyme, the human enzyme or mixed human and murine enzymes.

This figure shows the LDH analysis of 3 representative cell lines:-

Channel 1 - human breast homogenate, showing LDH enzyme band of human origin .

Channel 2 - UCT-Mel 7 cells from passage number 7' in vitro and passage number 9 in the nude mice, showing LDH enzyme band of human origin.

Channel 3 - UCT-Mel 7 cells from passage number 7' in vitro and passage number 7 in nude mice, showing LDH enzyme band of human origin.

Channel 4 - UCT-Mel 7 cells from passage number 8' in vitro and passage number 6 in the nude mice, showing LDH enzyme bands of human and mouse origin.

Channel 5 - UCT-Mel 7 cells from passage number 12' in vitro and passage number 6 in the nude mice, showing LDH enzyme band of mouse origin.

Channel 6 - mouse liver homogenate, showing LDH enzyme band of mouse origin.

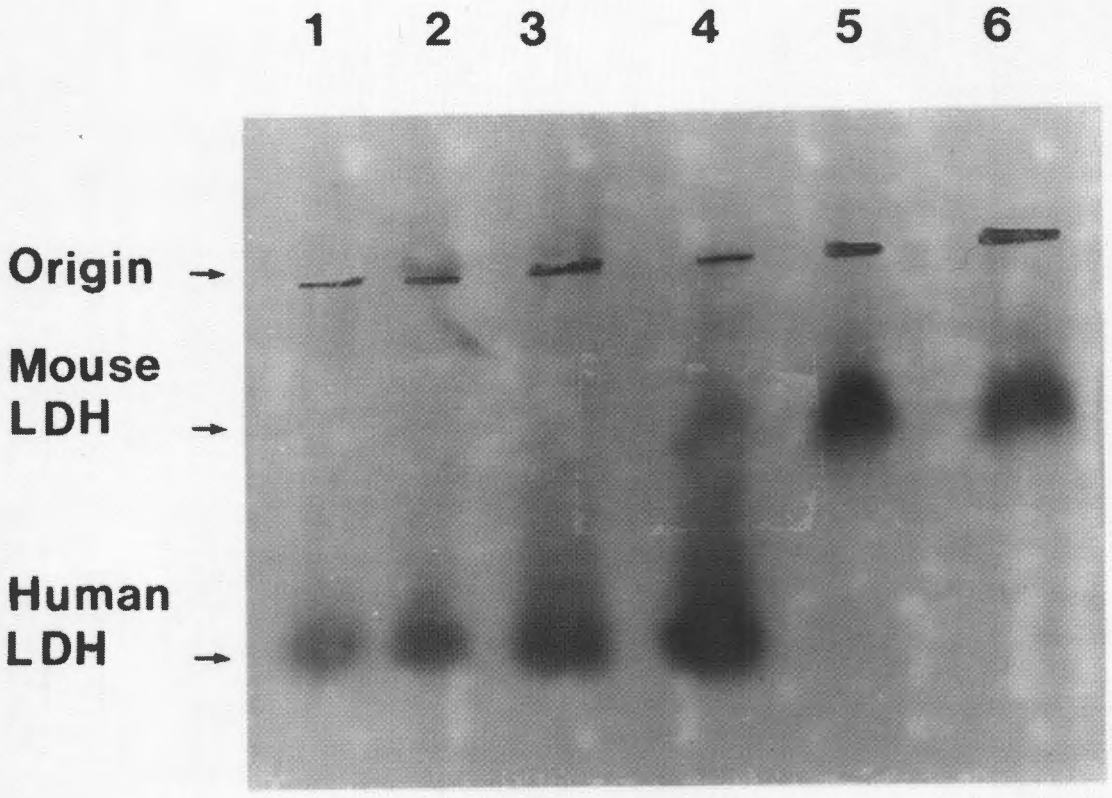


Figure 14

TABLE 2

Mouse	Passage No. in vivo									Passage No in vitro
	1	2	3	4	5	6	7	8	9	
NM-a										8'
LDH (H/M)	+++/+-**	+++/+	++/++	++/++	+++/+	+++/++*				++/+++
NM-b										12'
LDH (H/M)	+++/+	++/++	+++/+-	+++/+	+++/+	+++/+*				-/+++
NM-c										7'
LDH(H/M)	+++/+	+++/+	+++/+	+++/+	+++/+	+++/+	+++/-	++/++	+++/+*	+++/-
NM-d										15'
LDH(H/M)	+++/+	+++/+	+++/+	+++/+*						-/+++
NM-e										7'
LDH(H/M)	+++/+	++/++	+++/+	+++/+	+++/+	+++/+	+++/+*			+++/-

*These tumours were removed and placed in culture. Enzymes were determined at the indicated in vitro passage member.

**The relative intensities of the human/mouse LDH bands are indicated by the number of +.

DISCUSSION

The experiments that I report in this chapter have documented the growth and certain other characteristics of a xenografted human melanoma that behaved in a most unusual manner. By the study of this tumour I have been able to show, quite clearly, that a striking change in cellular phenotype occured during course of a prolonged period of repeated *in vitro* passaging. Subsequently, while resident as tumours in the mouse, the cellular phenotype changed even more dramatically.

Phenotypic drift of cell lines during the course of repeated *in vitro* passaging is well known, (Welch et al., 1983; Neri and Nicholson, 1981) and is, in most cases, reasonably ascribed to the operation of selective forces that favour the emergence of subpopulations with characteristics that confer survival and proliferative advantage in the *in vitro* milieu.

The differences that I observed, between the growth patterns of the 10th and 30th in vitro passage cells, would be entirely consistent with this process. Cells inoculated at the 10th passage grew, as do most melanomas, rapidly and progressively whereas 30' cells grew relatively slowly to reach a plateau size that was less than 1/10th of the volume that was possible for other melanomas to attain at a plateau where growth was inhibited by inadequate blood supply or nutrition. When fragments of the 10' cell-derived tumour were reimplanted into fresh recipients,the phasic growth pattern was seen. (Fig. 5) I interpret this observation as indicating that the early passage cell culture comprise two cell populations - those with the phasic growth phenotype and those that will grow normally. Repeated *in vitro* passage selected for the phasic growth cells so that, by the 26th passage these were the only cells that remained. It is of interest to note that one passage to the nude mouse also selected for survival, in the fresh recipient, of the cells that grew with a phasic pattern. (Fig. 5).

Although I have not yet completed enough experiments to be more convincing on this point, the in vivo growth pattern curve presented in (Fig. 3) suggests an extension of this interpretation since further evolution of the growth phenotype seemed to occur between the 30th and 56th in vitro passages. When 56' cells were inoculated, a barely palpable tumour resulted that stabilized at a size that was too small to document the growth, plateau or regression phases that characterized the phasic phenotype presented in Fig. 3a and b. Nonetheless, after remaining ostensibly dormant for 240 days, this tumour too suddenly escaped from the restraints upon its growth and a rapidly growing phase 5 tumour developed (Fig. 3c). If subsequent experiments confirm this observation, the following interpretation would best fit the finding. The UCT-Mel 7 line, as first established, contained 2 predominant populations that, for the sake of argument, I shall refer to as "r" (for rapid in vivo growth) and "s" (for slow in vivo growth).

I now suggest that cells belonging to population "r" have the characteristics of a "transit cell" population. (Aherne et al., 1977). - i.e. considerable but limited potential for rapid in vivo proliferation. Inocula of these cells would thus grow until the alotted number of divisions had been expended and then they would die. This would account for:

(i) the first three phases of the growth pattern depicted in Figs. 3a and b;

(ii) the paucity of r cells in the fragment transplanted into the fresh recipient described in Fig. 5.

I also suggest that the r cell population was marginally less well able to survive in vitro with the result that the phenotype was progressively lost as the in vitro passaging was continued from the 10' to the 56' stage.

The second, "s", population of cells comprised, I suggest, cells with the characteristics of stem cells that are endowed with the capacity for slow but nevertheless indefinite survival in vivo. The growth of tumours derived from

inocula of these cells was so slow that it was difficult to document the rate of increase in size of this population until a mutation occured in one of the "s" cell population or in a local murine stromal cell, at which point a rapidly growing, aggressive tumour developed. The two murine tumours that I observed (Table 2) testified to the mutagenic environment that would have obtained within the "s" cell tumour for such a mutation to occur. That the human phase 5 tumours were also composed largely of mutant cells is suggested by the fact that these cells grew rapidly and aggressively and transplanted into fresh recipients and showed none of the growth features of either "r" or the "s" cell parent from which they were putatively derived.

While this model probably represents an over simplification of the complex events that took place during the progression of the tumours from phase 4 to phase 5, I believe that it is useful for the fact that it suggests a number of further experiments that should be done and it provides a helpful basis for bringing my results into comparison with those of others who have studied the phenotypic changes that occur in experimental tumours during the course of their propagation in vivo or in vitro.

In the first place, if I am correct in my interpretation of the data, cloning of early passage cultures should enable me to derive pure populations of "r" and "s" cells. These should behave predictably in culture and the "r" cells should show senescence in vivo and the "s" cells not.

Secondly, I ought to be able to demonstrate other mutations in cells from the "s" cell tumours. This might be done by assaying the cells for resistance to cytotoxic agents such as thio-guanine or methotrexate using similar techniques to those used by others to detect mutant sub-populations in tumours (Yamashina et al., 1986). Alternatively it might be possible to detect oncogenic mutations in murine stromal cells by transplanting tumour fragments into immunocompetent nu/+ heterogotes. This latter experiment need not examine only phase 5 tumours; it would be most interesting, in fact, if

fragments of earlier phase tumours were tumourigenic in heterozygous animals since this could be used as the basis for experiments to obtain data on the length of time it was necessary for UCT-Mel 7 cells to be in contact with murine cells for effective conversion of the mouse stromal components to the malignant state.

Thirdly, it should be possible with this system, and experiments based upon the model that I have suggested, to identify the mutagenic influences that presumably operate in the tumours. Goldenberg and Pavia (1981; 1982) have also seen murine cell tumours develop in nude mice at the site of human xenografts and they favoured in vivo cell fusion of human tumour cells with normal host cells, with subsequent segregation of human chromosomes or of transforming "genes", as the basis for this horizontal transmission of malignancy. They produced no evidence, however, to support their preference for this idea. If this were the mechanism there should be no difficulty in demonstrating human genetic material (either by karyotypic analysis or by examining for the repetitive Alu sequences that occur every 10 kilobases in human DNA).

A prominent feature of late passage UCT-Mel 7 cells has been their ability to elicit an intense desmoplastic response accompanied by infiltration of the tumour with murine macrophages (Fig. 12). It is well-known that macrophages release factors that stimulate fibroblast proliferation (Hebbs et al., 1983) and numerous studies have documented the mutagenic activity of these cells in bacterial (Weitzman and Stossel, 1981; 1982; 1983) and mammalian cells (Weitberg et al., 1983; Yamashina et al., 1986). There is a good deal of circumstantial support for the belief that phagocyte-induced mutagenesis is mediated by the generation of active oxygen species that are characteristic products of this class of cells. Indeed, Weitzman, et al., (1985) have shown that stimulated neutrophils can cause malignant transformation of murine fibroblasts and Potter et al., (1985) have produced

evidence to support the suggestion that active oxygen species released by inflammatory cells serve as the mutagenic agents responsible for pristane-induced myelomas in Balb/c mice.

I have shown that macrophage infiltration is a predominent feature of phase 2 tumours and, in view of the published data I have cited, it seems likely that these cells were responsible for the progression of tumours to phase 5 and for the intense desmoplastic response that was seen. It is of interest to note, in this connection, that in the report by Goldenberg and Pavia, (1982) of a murine neoplasm arising at the site of a xenografted human malignancy, the histological appearances of the tumour that arose from the 10th passage of a human ovarian carcinoma through the nude mouse showed a heavy infiltration with fibroblastic elements. These features are similar to those that I noted with UCT-Mel 7 (Fig. 7).

If host macrophages do, indeed, play the important role that I have suggested, it should be possible to inhibit the in vivo progression of UCT-Mel 7 "s"-type cells by the administration, to the recipients, of anti-inflammatory agents such as indomethacin. Similarly one might be able to induce a change of "s" cells to the phase 5 phenotype by in vitro co-culture with macrophages alone, with macrophages activated by exposure to tumour promoters or with macrophages and fibroblasts. It will also be of interest to see if macrophages isolated from the tumour behave any differently, in this regard, than do those isolated from, for example, the peritoneal cavity.

In certain respects the model that I have proposed would accomodate the observations of Belin and Ossowski (1983) who found that Lewis lung carcinoma cells comprised two sub-types. The first and predominant type were cells that grew rapidly on the chicken CAM to produce tumours that were no longer tumorigenic in syngeneic mice. These cells would be analagous to the "r"-type cells that I propose. Their second cell population could be isolated by cloning the parental Lewis lung carcinoma culture. This yielded cells that

behaved differently in that they grew on the CAM and retained tumorigenicity. These would correspond to the "s" cells that I have postulated.

The effect of the growth environment on the malignant phenotype of the human HEp3 tumour was dramatically demonstrated by Ossowski and Reich, (1983). These cells grow as highly malignant tumours in chicken embryos. When transferred to in vitro culture, however, they showed a progressive loss of tumorigenicity that was essentially complete by the 40th passage. The aggressive malignant phenotype could, however, be recovered in these cells by prolonged exposure to the in vivo conditions of the CAM. Although, by virtue of the very different experimental system that was used by these authors, my results and those that they obtained are not entirely consonant, there are a number of points of similarity that are noteworthy. In the first place, the lose of tumorigenicity that they observed with prolonged in vitro culture resembled the transition that I found took place between the 10th and the 56th in vitro passage of UCT-Mel 7. The late passage cells required prolonged exposure (approximately 6 months) to "recover" their malignant potential (Fig. 3). Ossowski and Reich, (1983) were limited, by the embryonated egg system, to observation periods of 1 to 2 weeks in any given host; at the end of this time further observation of the tumour could only be secured by serial transfer onto a fresh CAM. As I have stated in the introduction, I was fortunate in that I was able to study the complete evolution of the slowly growing "s" cells in a single host.

There are a number of further respects in which the data I report merit comment.

It was interesting to observe, for example, the occasional emergence of a deeply pigmented phase 5 tumour from inoculated cells that were invariably amelanotic. Furthermore, although it was always easy to induce pigmented phase 5 tumours to re-establish themselves as permanent cell lines in culture, these lines were always non-pigmented. From these results it is possible to

draw two conclusions. Firstly, melanoma cells that have lost the capacity for melanin synthesis under one set of growth circumstances may recover that capacity when circumstances change. Common clinical experience tells us that malignant melanomas in man characteristically show areas of pigmentation and lack of pigmentation within the same cutaneous tumour. Indeed, this variegate appearance is a diagnostic hallmark of the disease. It is generally assumed that this heterogeneity reflects, primarily, a clonal change in the tumour cell population with the emergence of genotypic variants in which the ability to make melanin is irretrievably lost. My observations suggest that this is not always the case and that melanogenesis is recoverable in an amelanotic clone provided environmental circumstances are favourable.

I should also like to draw attention to the remarkable fact that tumours that grew very slowly in vivo proliferated quite rapidly in vitro (Fig. 6). This is consistent with the suggestion that I made earlier that "s" cells had an in vitro proliferative advantage over "r" cells, so that, by the 56th in vitro passage, essentially no "r" cells remained. Since I have not yet measured the doubling times of pure "r" cell populations, this comment is not strictly valid. I do, however, show data for phase 5 cells (Fig. 6) and these show that, despite their extremely rapid growth in vivo, their in vitro proliferation was sluggish.

Finally, it is perhaps worth mentioning once more the possible value of the UCT-Mel 7/nude mouse system for the study of the clonal evolution of human tumours.

Two excellent reviews have recently been published that consider this matter in detail. In the first, Schnipper (1986) considers the question of tumour-cell heterogeneity and rightly emphasises the considerable implications that within-tumour phenotypic variation has for the management of malignant disease in man. It is clear, from this scholarly account of the subject, that the author subscribes to the view that tumours are "malignant" in direct

proportion to the instability of their genetic apparatus and he leaves one with the sense that this instability is an innate feature of the neoplastic phenotype. The consistent chromosomal abnormalities that are encountered in such diseases as chronic myeloid leukaemia or Burkitt's lymphoma or the well known associations that exist between malignancy and Xeroderma pigmentosum or Bloom's syndrome certainly lend support to this view. I suspect, however, that interactions between tumour cells and the host environment may contribute more genetic change than is currently believed, and the experimental system I have described may well be used to vindicate this suspicion.

In the second paper Nowell (1986) has reviewed the subject of tumour progression and he, too, gives pride of consideration to the question of genetic instability. He does, however, consider host factors in clonal evolution and here he ascribes most importance to the immune system and the role of "immune surveillance" as forces that provide selective pressures that determine which mutant clones will expand to become the predominant subpopulation at any given time. The implication here is clear. First the mutation occurs and only then do host factors become important. I believe that UCT-Mel 7 will prove to be a useful model for showing that host reactions can generate mutants as well as eliminate them, and that the macrophage is not always the tumoricidal hero. It may play a villainous mutagenic role that is of far more consequence to the tempo at which malignant disease progresses.

APPENDIX

A.1 Tumour biopsies and primary culture:-

Samples were removed at the time of surgery and placed into bottles containing DB medium supplemented with 2% FCS and were immediately sent to the tissue culture laboratory.

The way in which this samples were treated depended on whether they were primary tumours in skin or whether they were metastatic deposits. Size and consistency of the specimen was taken into consideration too. Primary tumours were cultured as explants. Metastatic tumours were plated as single-cell suspensions after mechanical or enzymatic dispersion. When tissue was available, attempts were made to culture metastases as explants and primary tumours as single-cell suspension.

A.2 Culture of explants:-

The tissue was cut into small fragments (1 cu mm) with a scalpel blade very carefully not to damage the fragments by squeezing or disturbing them in any way. The pieces were placed as explants on 60 mm tissue culture dishes, moistened with a thin film of tissue culture medium and left overnight. The day after the explants were covered with medium and the cultures were then observed frequently for evidence of growth. The medium was changed once a week.

A.3 Mechanical dispersion:-

With sterile scissors, the specimen was minced finely, the tumour fragments suspended in RPMI medium and pipetted up and down 4 to 5 times through the orifice of a 5 ml pipette. The suspension was then allowed to stand for a few minutes during which time larger fragments settled and single cells remaining in suspension. Then the supernate was carefully aspirated into tubes and the single cells were sedimented by centrifugation at 350g for 5 min. The remained pellet was then resuspended in DB or RPMI medium supplemented with 10% FCS. This final suspension was placed into tissue culture dishes and observed regularly for evidence of growth.

A.4 Enzymatic dispersion:-

Cells that were retained in fragments that settled after mincing and pipetting were released by treatment with trypsin, followed where necessary by incubation with collagenase.

For trypsinization, the fragments were transferred to a bottle containing 0.25% trypsin in TD and gently agitated at 37°C for a maximum of 20 min. The digest was left to stand for 5 min. after which the supernatant was removed and added to an equal volume of medium containing 10% FCS to neutralise the proteolytic activity of the trypsin. The cell suspension was centrifuged at 350g for 5 min. Then the pellet was resuspended in growth medium and transferred to a tissue culture dish and incubated at 37°C in a humid atmosphere of 5% CO_2 in air.

This procedure was repeated for any remaining fragments.

After 3 or 4 sequential trypsin treatments any tumour tissue left undigested was incubated overnight at 4°C with collagenase at 1 mg/ml in RPMI-5% FCS

Occasionally a viscous and gelatinous suspension was observed following enzymatic digestion. This was the result of DNA release from damaged cells. DNAase, at a concentration of 10µg/ml was added to this gelatinous mixture which was then incubated at 37°C for 5-10 min. to give a fluid easily pipettable solution.

After 24 hours, all cultures were observed for viable cells. Petri dishes were washed gently with fresh medium to remove non-adherent cells which were pooled and centrifugated. The pellet was reseeded on to a new petri dish.

A.5 Maintenance and storage of cell lines:-

Once the cell lines were established, the cultures were maintained in Dulbecco's or RPMI medium supplemented with 10% FCS (heat-inactivated), 300 µg

penicillin/ml, 200 µg streptomycin sulphate/ml and 10µg tylocine/ml. The cultures were kept at 37°C in a humid atmosphere containing 5% CO_2 in air.

Cell lines cultured were examined at least twice weekly under phase contrast microscopy and were passaged when they approached confluence. Passaging was performed by aspirating the medium, and then covering the cells with 0.25% trypsin in TD at 37°C. After approximately 5 min, the detached cells were dispersed by gentle pipetting and the suspension was added to an equal volume of medium containing FCS to neutralize the protease. The cells were washed by centrifugation at 350g for 5 min, resuspended in medium, and adjusted to give the desired cell concentration for reseeding of new cultures.

To be preserved for long-term periods, cell stocks were frozen in liquid nitrogen. After centrifugation, the cell pellet was resuspended in tissue culture medium containing 10% FCS and 10% DMSO at room temperature. This suspension was distributed in 1 ml volumes into screw-topped 38x12.5mm nylon tubes (Nunc No. 1078) which were kept on chipped ice. These tubes were then frozen by vapor-phase liquid nitrogen freezing in a device built in the laboratory and designed to give programmed cooling at the rate of 4°C/min, until a temperature of -26°C was obtained. The cells were then held at this temperature for 20 min, after which they were cooled rapidly to -60°C and placed in liquid nitrogen. The freezing protocol proved to be extremely satisfactory (Farrant et al, 1974).

A.5.1 Cell lines

UCT-Mel 1. It was obtained from an inguinal metastasis of a 67 year old Caucasian female who initially had a primary malignant melanoma of Clark's level III removed from her right ankle. She died a year later with metastatic spread of the abdominal cavity.

The tissue sample for culture was a deeply pigmented lymph node which, on histological examination, showed partial or complete replacement by epithelioid malignant melanoma with melanin being its prominent feature. The

cells in tissue culture were non-pigmented at low density and they became deeply pigmented at confluence.

They grew in tissue culture with a doubling time of 41 hrs. The cells formed tumours in nude mice with doubling times of 6 days.

UCT-Mel 2 - Tumour tissue was obtained from a metastatic axillary lesion, removed from a 67 year old Caucasian female.

The sample for culture was macroscopically non-pigmented. The histological examination of the specimen confirmed the diagnosis of metastatic malignant melanoma. It was an epithelioid type cell and pigmentation was visible in certain areas, while others were non-pigmented.

Cells were initially passaged twice after which cell division ceased and they remained viable but dormant for 4 months. During this time medium was changed once a week. After 4 months of dormancy, cells started to divide simultaneously, in all ten 60 mm petri dishes.

The cells were non pigmented at low density, becoming deeply pigmented at confluence. They grew in vitro with a doubling time of 70 hrs (DB-FC10) and 50 hrs (RPMI-FC10) and in nude mice with doubling time of 5 days.

UCT-Mel 3 - This cell line was established from a metastatic liver deposit removed from a 71 year old Caucasian female. Histological examination of the tissue removed from the liver revealed metastatic malignant melanoma of epithelioid type. A Masson-Fontana silver stain was done, showing scanty intracellular melanin.

The cells were non pigmented at low density as well as at confluence. They grew in vitro with a doubling time of 58 hrs and in nude mice, with a doubling time of 8 days.

UCT Mel 4 - This cell line was established from a left inguinal lymph node

metastasis, removed from a 67 year old Caucasian female. The lymph node was macroscopically pigmented. Histology revealed a deeply pigmented metastsatic melanoma of epithelioid type. Cultured cells were non pigmented, even at high density, and had a doubling time in vitro of 52 hrs (RPMI-FC10) and 6 days in the nude mice.

UCT Mel 5 - Tumour tissue was obtained from a metastatic brain deposit removed from a 48 year old Caucasian female. The brain metastasis obtained for culture did not show melanin. Histological examination revealed it to be metastatic malignant melanoma of a mixed spindle and epithelioid cell type.

The cells in tissue culture were nonpigmented even at high density.

The doubling time in vitro was 58 hrs and 8 to 10 days in nude mice.

UCT-Mel 6 - This cell line was established from a metastatic malignant melanoma deposit removed from the left inguinal region of a 68 year old Caucasian female.

The sample for tissue culture was non-pigmented. The histological report confirmed the diagnosis of metastatic malignant melanoma, of spindle cell type, with no melanin pigment present.

The cells in tissue culture were non pigmented even at high density.

The doubling time in tissue culture was 33 hrs and this tumour failed to grow in the nude mice.

UCT-Mel 7 - This cell line was established in tissue culture in May 1980, from from a secondary malignant melanoma removed from the left femoral gland of a 52 year old negro female.

The sample for culture was non-pigmented. Histological examination showed secondary malignant melanoma of spindle cell morphology and minimal production of pigment.

The cells in culture were non-pigmented, even at high density.

The doubling time *in vitro* was 52 hrs and 9 days in nude mice.

These cell lines were studied *in vitro* and characterized in more detail by E. Hoal, 1981, in her Ph.D thesis and Hoal-van Helden (1986).

A.6 Mycoplasma contamination

All the cell lines used were tested for mycoplasma contamination. The method used was that described by Chen (1977). This involved staining cultures with a DNA-specific fluorescent stain.

Cells were cultured for 7 days in medium from which tylocine had been omitted. Cells were then fixed with acetic acid:methanol (1:3) and stained with bisbenzimide fluorochrome (Hoechst No. 33258) at 0.5 ug/ml in Hank's balanced salt solution for 30 min. The cell layer mass was well rinsed in deionized water and mounted in buffer containing 0.02M citric acid, 0.06 M disodium phosphate and 50% glycerol, pH 5.5. Cultures were examined with a Leitz fluorescence microscope.

TABLE A.7

ORIGIN AND CHARACTERISTICS OF MELANOMA CELL LINES

CELL LINE	SITE OF BIOPSY	PIGMENTATION IN BIOPSY	PIGMENTATION IN VITRO	PIGMENTATION IN MOUSE	GROWTH IN SOFT AGAR	GROWTH IN NUDE MOUSE
UCT-Mel 1	R.Inguinal L. node	+	+	+	+	+
UCT-Mel 2	L.Axillary L. node	±	+	±	+	+
UCT-Mel 3	Liver	-	-	-	+	+
UCT-Mel 4	L.Inguinal L. node	+	-	-	-	+
UCT-Mel 5	Brain	-	-	-	+	+
UCT-Mel 6	L.Inguinal L. node	-	-	-	-	-
UCT-Mel 7	L.Femoral gland	-	-	-	+	+

TABLE A.8

CELL LINE/ ORIGIN	NO OF CELLS ($x10^5$)	NO. OF PASSAGES IN VIVO	NO. OF PASSAGES IN VITRO	α (day^{-1})	β (day^{-1})	Vmax (mm^3)	Td(days) ($V=50mm^3$)	Td(days) ($v=200mm^3$)	Td(days) ($V=1000mm^3$)
UCT-Mel 1	-	4	-	0.126	9.06^{-3}	$2.5x10^8$	5.1	5.6	6.3
(Lung met	-	7	-	0.107	6.66^{-3}	$3.0x10^9$	5.9	6.4	7.1
passaged s.c.	-	12	-	0.054	5.35^{-2}	$2.9x10^3$	3.5	5.6	20.0
UCT-Mel 2	4	-	124'	0.125	1.54^{-3}	-	5.6	5.7	5.8
(Parent cells)	10	-	70'	0.121	4.89^{-3}	$3.8x10^{11}$	6.3	6.7	7.3
(Lung met	-	6	-	0.147	7.38^{-2}	$2.0x10^4$	2.8	4.8	82.8
passaged s.c.	-	9	-	0.206	9.51^{-2}	$1.5x10^4$	1.4	1.8	3.1
	-	10	-	0.270	2.64^{-2}	$1.3x10^5$	3.5	4.3	5.8
UCT-Mel 3	10	-	69'	0.166	1.73^{-2}	$4.2x10^5$	4.6	5.5	7.0
(Parent cells)	10	-	87'	0.241	2.80^{-2}	$2.8x10^4$	4.1	5.4	8.3
(Lung met	-	1	-	0.145	4.09^{-2}	$1.0x10^4$	3.1	4.8	8.8
passaged s.c.	-	16	-	0.127	2.09^{-2}	$3.2x10^5$	3.9	4.7	6.1
	-	25	-	0.139	6.20^{-3}	-	4.5	4.7	5.1
	10	6	12'	0.171	2.17^{-2}	$3.8x10^3$	8.0	12.4	33.8
	20	11	3'	0.199	2.58^{-2}	$1.1x10^5$	3.7	4.5	6.2
	30	11	11'	0.139	6.67^{-3}	$9.2x10^9$	5.6	6.0	6.6
UCT-Mel 4	-	1	-	0.157	6.50^{-2}	$8.3x10^4$	2.2	3.2	6.1
(Lung met	-	8	-	0.124	1.70^{-2}	$2.8x10^6$	3.8	4.4	5.3
passaged s.c.	-	15	-	0.242	1.79^{-2}	$6.1x10^8$	2.4	2.6	3.0
UCT-Mel 5	-	2	-	0.062	4.41^{-3}	$2.8x10^8$	10.3	11.4	12.9
(Lung met	-	7	-	0.058	4.26^{-4}	-	11.9	12.1	12.2
passaged s.c	-	10	-	0.215	2.01^{-2}	$1.0x10^6$	3.6	4.2	5.2
	10	5	5'	0.118	2.05^{-2}	$9.8x10^4$	6.9	9.6	17.6

TABLE A.9

CELL LINE	NO. OF CELLS ($\times 10^5$)	HORMONAL TREAT.	NO. OF TUMOURS/NO. OF MICE MALES CAST	MALES NORMAL	FEMALES OVAR.	FEMALES NORMAL	DELAY TIME $100mm^3$	α (day^{-1})	β (day^{-1})	Vmax (mm^3)	Td (days) $V=50mm^3$	Td (days) $V=200mm^3$	Td (days) $V=1000mm^3$
ET-Mel 1	10	-	5/5				23.7	0.250	3.17^{-2}	2.8×10^4	3.7	4.8	7.4
	10	-		5/5			25.1	0.207	1.78^{-2}	1.2×10^6	4.0	4.7	5.8
	10	-			5/5		25.8	0.186	2.32^{-2}	3.4×10^4	4.8	6.3	9.5
	10					5/5	25.4	0.203	2.99^{-2}	9.4×10^3	4.7	6.6	12.3
	10	DHT	5/5				27.8	0.171	1.62^{-2}	4.5×10^5	4.9	5.8	7.4
	10	DHT		5/5			28.2	0.168	1.16^{-2}	2.1×10^7	4.8	5.3	6.2
	10	DHT			5/5		28.5	0.174	2.10^{-2}	4.0×10^4	5.2	6.7	9.9
	10	DHT				5/5	23.1	0.194	2.43^{-2}	5.2×10^4	4.3	5.5	7.9
	10	E2	5/5				20.8	0.187	2.12^{-2}	2.0×10^5	4.1	5.0	6.7
	10	E2		5/5			24.3	0.245	2.34^{-2}	3.1×10^5	3.5	4.2	5.4
	10	E2			5/5		19.5	0.243	4.12^{-2}	9.2×10^3	3.5	4.8	9.1
	10	E2				5/5	21.6	0.196	7.54^{-2}	—	3.7	3.9	4.2
	10	-	Not done	3/5			20.3	0.179	4.59^{-2}	1.3×10^4	5.2	9.9	-
	10	-				4/5	18.6	0.163	3.92^{-2}	6.2×10^3	4.0	5.7	12.1
	10	E2	Not done	5/5	Not done		20.7	0.159	1.09^{-2}	5.8×10^7	4.7	5.2	6.0
	10	E2				5/5	19.9	0.215	2.55^{-2}	8.8×10^4	3.8	4.7	6.6
	10	TAM+E2				5/5	16.3	0.211	1.83^{-2}	4.1×10^6	3.5	4.0	4.8
	10	TAM				5/5	19.8	0.208	3.03^{-2}	2.1×10^4	4.0	5.3	8.5
	10	PROG				5/5	20.0	0.220	3.85^{-2}	6.2×10^3	4.0	5.8	12.4
	10	PROG+E2				5/5	20.2	0.150	3.16^{-2}	1.1×10^4	4.3	6.0	10.7

TABLE A.10

CELL LINE	NO. OF CELLS ($x10^5$)	HORMONAL TREAT.	NO. OF TUMOURS/NO. OF MICE MALES CAST	MALES NORMAL	FEMALES OVAR.	FEMALES NORMAL	DELAY TIME $100mm^3$	α (day^{-1})	β (day^{-1})	Vmax (mm^3)	Td(days) $V=50mm^3$	$V=200mm^3$	$V=1000mm^3$
UCT-Mel 2	1	-	0/4				-	-	-	-	-	-	-
	1			4/4			97	0.120	8.12^{-3}	$2.5x10^7$	6.7	7.5	8.7
	1				2/5		48	0.229	0.03	$1.4x10^5$	3.5	4.2	5.7
	1					2/5	66	0.330	9.40^{-2}	$9.0x10^2$	2.9	6.6	-
	1.5	-	0/5				-	-	-	-	-	-	-
	1.5			2/5			58.8	0.185	3.19^{-2}	$1.3x10^3$	7.4	14.3	-
	1.5	DHT	2/5				76.8	0.235	5.97^{-2}	$1.9x10^2$	2.5	-	-
	2		3/5				59.3	0.246	3.06^{-2}	$2.3x10^4$	3.9	5.2	8.2
	2			1/5			40.1	0.169	0.03	$2.5x10^3$	7.3	12.0	52.0
	2				1/5		40.7	0.141	8.96^{-4}	$0.3x10^1$	5.0	5.0	5.1
	2					1/4	50.1	0.307	4.34^{-2}	$2.9x10^3$	4.3	6.9	24.3
	2	DHT	3/5				43.7	0.393	6.80^{-2}	$3.6x10^2$	6.4	-	-
	2			2/5			57.9	0.281	7.32^{-2}	$1.4x10^2$	5.0	15.0	4.1
	2				5/5		57.2	0.093	4.03^{-3}	$3.6x10^{10}$	8.61	9.2	10.1
	2					2/5	53.8	0.227	3.74^{-2}	$1.6x10^3$	5.9	10.8	-
	4		4/5				31.8	0.222	3.06^{-2}	$1.9x10^4$	4.0	5.4	8.7
	4			3/5			67.7	0.153	1.68^{-2}	$1.2x10^5$	5.5	6.8	9.2
	4				3/4		38.8	0.129	1.08^{-2}	$4.7x10^6$	5.8	6.6	7.9
	4					1/5	37.0	0.126	1.54^{-3}	$0.6x10$	5.6	5.7	5.8
	4	DHT	4/4				31.0	0.333	7.01^{-2}	$6.3x10^2$	4.6	13.2	13.1
	4			3/4			42.8	0.204	4.60^{-2}	$1.4x10^3$	5.1	9.5	-
	4				4/5		35.5	0.139	1.04^{-2}	$6.7x10^5$	5.8	6.6	17.9
	4					3/5	38.3	0.156	9.42^{-3}	$8.1x10^7$	5.3	5.9	6.7
	4	E2	5/5				26.9	0.127	1.882^{-2}	$0.2x10$	5.6	5.7	5.9
	4			5/5			28.3	0.179	9.410	$3.4x10^9$	4.2	4.5	5.0
	4				4/4		25.9	0.155	0.029	$3.1x10^3$	6.2	9.8	31.0
	4					4/5	30.0	0.155	1.55^{-2}	$1.0x10^6$	4.7	5.4	6.8
	10		Not done	Not done	5/5		32.1	0.189	3.48^{-3}	$0.2x10$	5.9	10.0	76.9
	10					5/5	35.5	0.156	2.37^{-2}	$6.0x10^3$	6.6	9.6	20.6
	10	E2			5/5		27.4	0.155	1.96^{-2}	$5.2x10^4$	5.3	6.8	9.8
	10	E2				5/5	30.6	0.148	2.07^{-2}	$9.3x10^3$	6.9	9.6	12.9
	10	TAM			5/5		28.6	0.181	2.62^{-2}	$5.0x10^3$	6.0	8.7	18.9
	10	TAM				5/5	25.8	0.111	2.05^{-4}	-	6.2	6.2	6.3
	10	TAM+E2			5/5		26.7	0.134	2.91^{-3}	-	5.3	5.5	5.7
						5/5	22.2	0.194	0.034	$3.5x10^3$	5.2	8.1	23.8

TABLE A.11

CELL LINE	NO. OF CELLS ($x10^5$)	HORMONAL TREAT.	NO. OF TUMOURS/NO. OF MICE MALES CAST	MALES NORMAL	FEMALES OVAR.	FEMALES NORMAL	DELAY TIME $100mm^3$	α (day^{-1})	β (day^{-1})	Vmax (mm^3)	Td(days) V=50mm^3	V=200mm^3	V=1000mm^3
UCT-Mel 3	10				5/5		31.6	0.120	2.19^{-3}	$0.5x10^1$	6.01	6.2	6.4
	10					5/5	36.3	0.148	1.03^{-2}	$8.1x10^6$	5.81	6.6	7.8
	10	DHT			4/4		33.1	0.082	9.68^{-3}	$1.0x10^5$	9.8	12.1	16.7
	10					5/5	27.9	0.111	6.58^{-4}	0.5x10	6.3	6.3	6.4
	10	E2			5/5		27.6	0.144	1.25^{-2}	$1.4x10^6$	5.61	6.5	8.1
	10					5/5	29.2	0.135	6.91^3	$3.6x10^9$	5.71	6.1	6.8
	10		3/5				41.3	0.188	9.25^{-2}	$2.6x10^3$	2.11	3.4	13.1
	10			5/5			37.8	0.114	8.08^{-3}	$1.6x10^8$	5.9	6.5	7.4
	10				5/5		42.8	0.164	1.39^{-2}	$8.7x10^6$	4.21	4.8	5.7
	10					5/5	39.9	0.155	4.44^{-3}	$0.4x10^1$	4.6	4.8	5.0
	10	DMT	4/5				39.6	0.123	4.68^{-3}	$0.4x10^1$	6.0	6.3	6.8
	10			5/5			35.8	0.137	2.74^{-2}	$3.9x10^3$	6.3	9.7	25.9
	10				5/5		29.8	0.211	2.75^{-2}	$1.2x10^4$	4.9	6.7	11.6
	10					5/5	28.9	0.160	2.14^{-2}	$4.2x10^4$	5.1	6.5	9.6
	10	E2	5/5				31.2	0.147	9.74^{-3}	$7.8x10^7$	5.1	5.7	6.6
	10			5/5			33.4	0.207	3.63^{-2}	-	3.4	4.4	7.1
	10				5/5		24.7	0.252	4.68^{-2}	$6.7x10^3$	3.3	4.7	9.7
	10					5/5	34.2	0.141	2.26^{-2}	$3.5x10^4$	4.7	6.4	9.6
	10				4/5		37.8	0.133	6.75^{-3}	$1.1x10^{10}$	5.5	5.9	6.5
	10					4/5	32.4	0.193	0.02	$9.9x10^4$	4.6	5.7	7.9
	10	DHT			5/5		34.9	0.139	2.07^{-2}	$4.9x10^4$	5.1	6.5	9.4
	10					5/5	34.9	0.150	2.83^{-2}	$1.1x10^4$	4.9	6.8	12.2
	10	E2			5/5		32.1	0.138	6.61^{-3}	$1.8x10^{10}$	5.4	5.8	6.4
	10					5/5	33.5	0.150	2.94^{-3}	-	4.7	4.8	5.0
	10	TAM+E2			5/5		26.2	0.161	2.10^{-2}	$5.1x10^4$	5.0	6.3	9.2
	10					5/5	26.8	0.127	5.14^{-3}	-	5.4	5.8	6.2
	10	DHT+E2			4/5		40.6	0.150	4.76^{-2}	$7.4x10^2$	6.3	16.0	-
	10					5/5	32.0	0.159	1.50^{-2}	$4.5x10^5$	5.3	6.2	8.0

TABLE A.12

CELL LINE	NO. OF CELLS	HORMONAL TREAT.	NO. OF TUMOURS/NO. OF MICE MALES		FEMALES		DELAY TIME	α	β	Vmax	Td(days)		
	($x10^5$)		CAST	NORMAL	OVAR.	NORMAL	$100mm^3$	(day^{-1})	(day^{-1})	(mm^3)	$V=50mm^3$	$V=200mm^3$	$V=1000mm^3$
UCT-Mel 7	10		5/5					4.61^{-3}	9.20^{-4}	-	-	93.9	78.2
	10			4/4			586.8	4.70^{-3}	9.10^{-4}	$2.1x10^3$	223.2	337.8	-
	10				6/6		-	4.25^{-2}	2.96^{-2}	$0.4x10^2$	-	-	-
	10					5/5		5.75^{-2}	7.42^{-2}	0.3x10	-	-	-
	10	DHT	5/5				61.8	4.38^{-2}	5.69^{-3}	$4.0x10^4$	19.2	24.6	36.5
	10			5/5			-	0.13	0.07	$7.9x10^1$	-	-	-
	10				5/5		117.4	3.70^{-2}	1.12^{-2}	$2.7x10^2$	47.0	-	-
	10					4/5	79.9	5.64^{-2}	1.04^{-2}	$1.4x10^3$	22.5	42.7	.
	10	E2	5/5				51.5	8.47^{-2}	1.71	$1.2x10^3$	14.5	19.3	-
	10			5/5			102.3	2.58^{-2}	4.3	$5.7x10^3$	36.7	53.7	117.7
	10				5/5		56.9	7.52^{-2}	2.12^{-2}	$3.8x10^2$	19.8	-	-
	10					5/5	62.8	4.30^{-2}	2.13^{-3}	$7.3x10^9$	17.7	19.1	21.1

TABLE A.13

UCT-MEL 5 AND METASTASIC SELECTION

CELL LINE/ORIGIN	NO. OF MICE	PASSAGE NO. IN MICE	TUMOUR WT. AT REMOVAL (gm)	AV. LENGTH OF TIME TO METASTASES	METASTASES %	METASTASES SITE
UCT-Mel 5	22	-	0.5; 0.8; 1.2; 1.5; 1.7; 2.6; 2.9	230	14	Lung
UCT-Mel 5 (Lung met. passaged s.c)	24	1 - 4	0.4; 0.6; 0.8; 1.1; 1.6; 1.9; 2; 2.3; 2.8; 3.8; 5.3; 5.5	150	67	Lung Kidney Somach Lung Uterus
	10	5 - 6	0.9; 2.0; 2.2; 2.5; 2.6; 2.7; 3.1; 5.1	180	80	
	8	7 - 10	0.9; 1.3; 1.9; 3.1; 3.4; 4.1; 6.9; 7.6	150	100	Lung

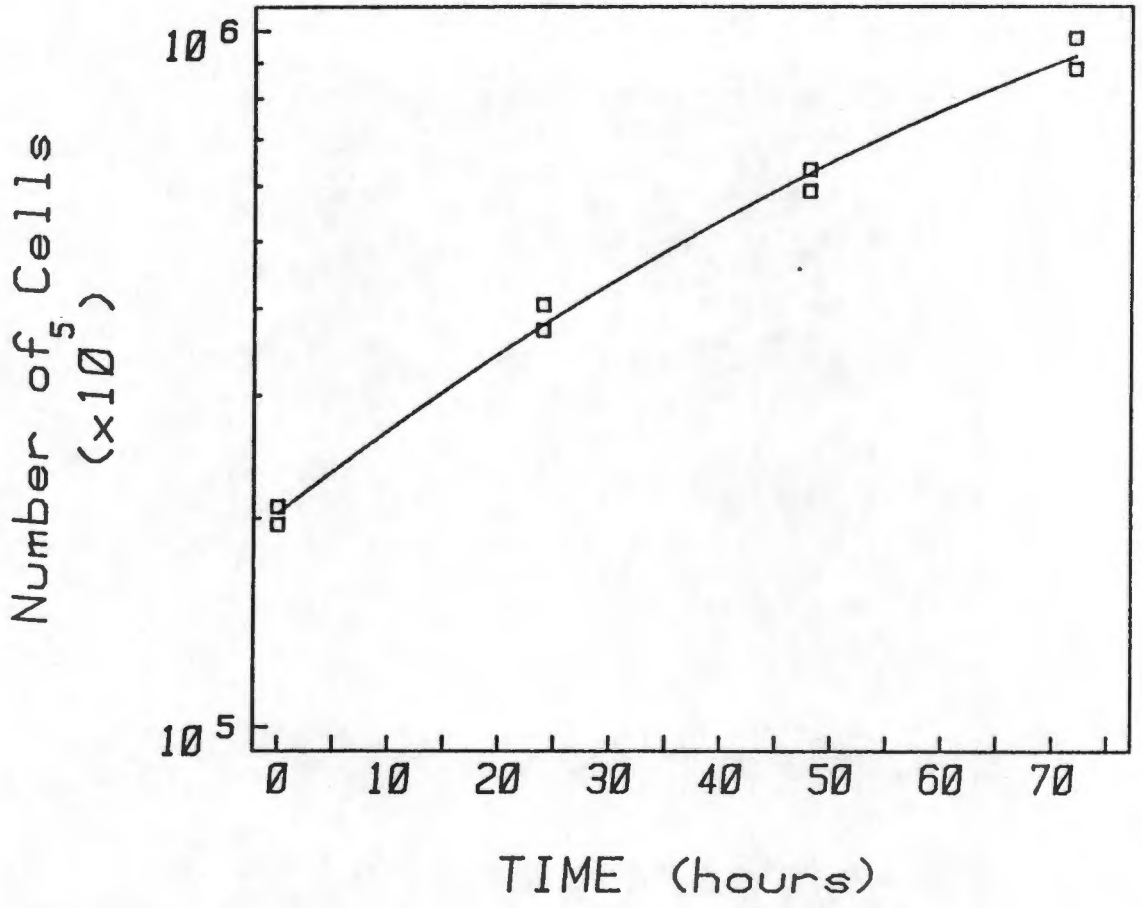


Figure A.14

Growth curve of UCT-Mel 5 cells in vitro

A lung metastasis from a mouse inoculated subcutaneously with $5x10^6$ UCT-Mel 5 cells (27') was sequentially passaged through nude mice. The tumour was removed at the 5th in vivo passage and cultured in vitro for 12 passages. The cells were seeded ($2x10^5$ cells) in RPMI-FC10 in 35mm dishes. Twenty four hr after seeding, the medium was aspirated and fresh RPMI-FC10 was added to the cultures. Thereafter the culture medium was changed every 48 hr. At the indicated times in the figure, replicate cultures were trypsinized and the cells were counted in a Coulter counter.

The generation time of the cells was 33 hr.

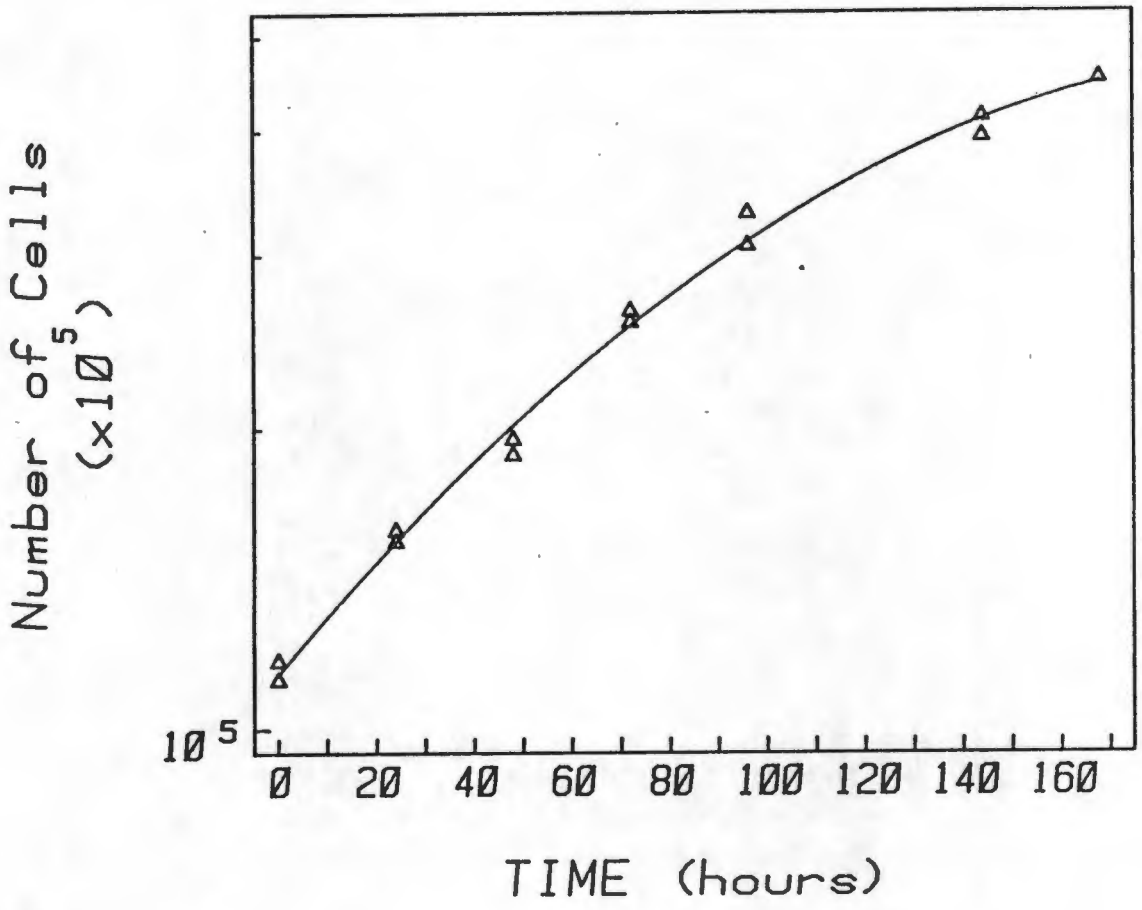


Figure A.15

Growth curve of UCT-Mel 3 cells in vitro

A metastatic lung deposit from a mouse inoculated subcutaneously with 5×10^6 UCT-Mel 3 cells (69'), was sequentially passaged in vivo in nude mice. The tumour removed at the 6th passage in vivo mass cultured in vitro for 11 passages. The cells (2×10^5 cells) were seeded in RPMI-FC10 on 35mm dishes. Twenty four hr after seeding, the medium was aspirated and fresh RPMI-FC10 was added to the cultures. Thereafter, the culture medium was changed every 48 hr. At the times indicated on the graph, replicate cultures were trypsinized and the cells were counted in a Coulter counter.

Note that a period of exponential growth was followed by a diminished growth rate as higher saturation densities are reached.

The generation time of the cells during the exponential phase of growth was 86 hours.

Table A.16

Phase of growth	Collagen Content (ng hydroxyproline/μg protein)
Phase 2	51.5; 37.2; 123.1; 80.0; 79.4; 51.2
Phase 3	2.8; 3.8; 2.7; 1.1
Phase 5P	2.2(P1); 2.9(P1); 1.2(P1); 0(P16); 0(P8) 4.5(P6); 1.5(P18); 0.(P1); 0(P12); 0(P14) 0(P1); 0(P17)

Each value represents the hydroxyproline content of an individual tumour. The number in parenthesis indicates the in vivo passage number of the tumour.

Table A.17

UCT-Mel 7 Cell Line Passage in Nude Mice

Origin	No.of Mice / No.of Tumour	No.of Passage in vitro	No.of Passage in vivo	Delay Time ($100mm^3$) (days)	α (day^{-1})	β (day^{-1})	Vmax (mm^3)	T/d days (V=50mm)	T/d days (V=200mm)	T:2 in vitro (hrs)
Parent	5/5	8	-	30.9	0.103	1.54^{-2}	$1.4x10^4$	8.5	11.5	95
	5/5	10	-	29.6	0.213	4.54^{-2}	$1.4x10^9$	5.1	9.6	-
	5/5	10	-	32.1	0.101	2.31^{-2}	$5.4x10^3$	-	-	-
	5/5	26	-	30	0.420	2.34^{-2}	$3.2x10^2$	19.8	-	-
	5/5	30	-	14.4	0.107	5.97^{-2}	$8.8x10^2$	-	-	52
	5/5	52	-	*	0.260	1.12^{-2}	7.1x10	-	-	47
Phase 5	5/5	8	1	32.3	0.242	0.70^{-3}	$9.3x10^3$	21.1	29.5	-
	5/5	10	8	30.3	0.122	1.60^{-3}	-	5.8	6.0	282
	5/5	10	10	29.0	0.904	1.01^{-3}	-	7.8	7.9	141
	5/5	30	1	74.9	0.115	3.48^{-2}	$2.3x10^3$	5.7	9.6	-
	5/5	30	1	48.3	0.772	3.36^{-3}		9.7	10.3	-

*This tumour never reached the volume of 100 mm^3

REFERENCES

Acton R.T., Balch C.H., Barger B.O., Dudlowle B., Go R.C.P., Soong S-J and Roseman J.M. (1983). The occurrence of melanoma and its relationship with host, lifestyle and environmental factors. In: Malignant Melanoma 1, Constanzi J.J. (ed.) The Hague, Nijhoff. pp. 151-182.

Adam S.A., Sheares J.K., Wright N.H., Mosser G., Harris R.W. and Vessey M.P. (1981). A case-control study of the possible association between oral contraceptives and malignant melanoma. Brit. J. Cancer 44: 45-50.

Aherne W.A., Camplejohn R.S. and Wright, N.A. (1977). An introduction to cell population kinetics. Edward Arnold, London.

Allen A.C. and Spitz S. (1953) Malignant melanoma. A clinicopathological analysis of the criteria for diagnosis and prognosis. Cancer 6: 1-45.

Allen E.P. (1955). Malignant Melanoma. Spontaneous regression after pregnancy. Br. Med. J. i: 647-655.

Amer M.H., Al-Sarraf M. and Vaitkevigvs V.K. (1979). Clinical presentation, natural history and prognostic factors in advanced malignant melanoma. Surg. Gynecol. Obstet. 149: 687-692.

Anderson D.E. (1971) Clinical characteristics of the genetic variety of cutaneous melanoma in man. Cancer 21: 721-725.

Atkison P.R., Weidman E.R., Bhavmick B. and Bala R.M. (1980). Release of somatomedin-like activity by cultured WI-38 human fibroblasts. Endocrinology 106: 2006-2012.

Ashley D.J.B. (1965). On the incidence of carcinoma of the prostate. J. Pathol. Bacteriol. 90: 217-224.

Aubert C., Chiriecleanu E., Foa C., Rorsman H., Rosengren E. and Rouge F. (1976). Ultrastructural and biochemical changes in cultured human malignant melanoma cells after heterotransplantation into nude mice. Cancer Res. 36: 3106-3112.

Aubert C., Rouge F. and Galindo J.R. (1980). Tumorigenicity of human malignant melanocytes in nude mice in relation to their differentiation in vitro. J. Natl. Cancer Inst. 64: 1029-1040.

Azar H.A., Hansen C.T. and Costa J. (1980). N:NIH(S)II-nu/nu mice with combined immunodeficiency: a new model for human tumour heterotransplantation. J. Natl. Cancer Inst., 65(2): 421-430.

Balch C.M., Wilkerson J.A., Murad T.M., Soong S., Ingalls A.L. and Maddox W.A. S (1980). The prognostic significance of ulceration of cutaneous melanoma. Cancer 45: 3012-3017.

Balch C.M., Soong S.J., Murad T.M., Ingalls A.L. and Maddox W.A. (1983). A multifactorial analysis of melanoma. IV Prognostic factors in 200 melanoma patients with distant metastases (Stage III). J. Clin. Oncol. 1: 126-134.

Baldwin R.W. and Pimm M.L. (1980). Human tumour xenografts in athymic nude mice: non-specific host rejection responses. In: Immunodeficient Animals for Cancer Research. MRC Laboratory Animal Centre Symposium Number 2. (ed.) S. Sparrow. The MacMillan Press Ltd., New York/London pp 125-133.

Barak M., Ulitzur S. and Merzbach D. (1983) Phagocytosis-induced mutagenesis in bacteria. Mutat. Research 121: 7-16

Barlogie B., Drewinko B., Schumann J., Gohde W., Dosik G., Latreille J., Johnston D.A. and Freireich E.J. (1980). Cellular DNA content as a marker of neoplasia in man. Am. J. Med. 69: 105-203.

Bastert G.B., Fortmeyer H.P. and Schmidt-Matthiesen H.(1981).(eds.) Thymusaplastic nude mice and rats in clinical oncology. Gustav Fischer, Stuttgart, New York.

Beattie C.W., Chaudhurt P.K., Walker M.J. and Gupta T.K. (1979). Influence of sex on the rate of growth of human malignant melanoma. Am. Assoc. Cancer Res. 20: 130.

Beckwith J.B. and Perrin E.V. (1963). In situ neuroblastomas: a contribution to the natural history of neural crest tumors. Am. J. Pathol. 43: 1039.

Belin D. and Ossowski L. (1983) Loss of tumourigenicity of Lewis lung carcinoma on the chorioallantoid membrane of the chick embryo. Cancer Res. 43: 3263-3269.

Bellet R.E., Danna V., Mastrangelo M.J. and Berd D. (1979). Evaluation of a "nude" mouse-human tumour panel as a predictive secondary screen for cancer chemotherapeutic agents. J. Natl. Cancer Inst. 63(5): 1185-1188.

Beral V., Ramcharan S. and Fabis, R. (1977). Malignant melanoma and oral contraceptive use among women in California. Br. J. Cancer 36: 804-809.

Beral V., Evans S., Shaw H. and Milton G. (1983). Cutaneous factors related to the risk of malignant melanoma. Br. J. Dermatology 109: 165-172.

Bodenham D.C. and Hale B. (1972). Malignant melanoma. Endocrine therapy in malignant disease. (ed.) by B.A. Stoll, London. W.B. Saunders Co. Ltd. pp. 377-383.

Bronson F.H. (1971). Rodent pheromones. Biology of Reproduction 4: 344-357.

Brooks C.G., Webb P.J., Robins R.A., Robinson G., Baldwin R.W. and Festing M.F.W. (1979). The immunology of Rnu/Rnu "nude" rats with congenital aplasia of the thymus. Eur. J. Immunol. 10: 58-65.

Brünner N. and Visfeldt J. (1982). Histologic changes following oestradiol treatment of a hormone-responsive human breast carcinoma grown in nude mice. Acta Path. Microbiol. Immunol. Scand. Sect. A, 90: 355-362.

Buck R.C. (1973). Walker 256 tumor transplantation in normal and injured peritoneum studied by electron mciroscopy, scanning E.M. and radiocultography. Cancer Res. 33: 3181-3188.

Burk R.R. (1973). A factor from a transformed cell line that affects migration. Proc. Natl. Acad. Sci. U.S.A. 70: 369-371.

Burrows M.T. (1914). The growth of human malignant tumours in vitro. Medical Record 86: 649.

Byrd B.F. and McGanity W.J. (1954). The effect of pregnancy on the clinical course of malignant melanoma. South Med. J. 47: 196-200.

Carr I. and Carr J. (1982). Experimental models of lymphatic metastasis. In: Tumor Invasion and Metastasis, (eds.) Liotta L.A. and Hart I.R., Development in Oncoloy 7. Martinus Nijhoff Publishers pp. 189-206.

Carrel S., Sordat B. and Merenda C. (1976). Establishment of a cell line (Co-115) from a human colon carcinoma transplanted into nude mice. Cancer Res. 36: 3978-3984.

Chaudhuri D.K., Walker M.J., Beattie C.W. and Dasgupta T.K. (1979). Endocrine correlates of human malignant melanoma. J. Surg. Res. 26: 214-219.

Chen T.R. (1977). In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell Res. 104: 255-262.

Chew E.C., Josephson R.L. and Wallace A.C. (1976). Morphologic aspects of the arrest of circulating cancer cells. Fundamental Aspects of Metastases. (ed.) L. Neiss, pp. 121-150.

Chung A.E., Jaffe P., Freeman I.L., Vergens J-P., Braguiski J.E. and Carlin B. (1979). Properties of a basement membrane related glycoprotein synthesized in culture by a mouse embryonal carcinoma derived line. Cell 16: 277-281.

Clarke C.L. and Satyaswaroop P.G. (1985). Photo-affinity labeling of the progresterone receptor from human endometrial carcinoma. Cancer Res. 45: 5417-5420.

Clayton S.G. (1946). Melanoma of vulva with pregnancy. Proc. R.Soc. Med. 39: 578-579.

Clemmons D.R., Underwood L.E. and van Wyk J.J. (1981). Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. J. Clin. Invest. 67: 10-19.

Cleveland W.S. and McGill R. (1985). Graphical perception and graphical methods for analyzing scientific data. Science 229: 828-833.

Clifton E.E. and Agostino D. (1962). Factors affecting the development of metastatic cancer effects of alterations in clotting mechanism. Cancer 15: 276-283.

Cloudman A.M. (1941). Special articles. The effect of an extra-chromosomal influence upon transplanted spontaneous tumors in mice. Science 93: 380.

Cochran A.J. (1969). Malignant Melanoma. Cancer 23: 1190-1199.

Cole W.H. (1974). Spontaneous regression of cancer. The metabolic triumph of the host? Ann. N.Y. Acad. Sci. 230: 111-141.

Coman D.R. (1961). Adhesiveness and stickiness: two independent properties of the cell surface. Cancer Res. 21: 1436-1438.

REFERENCES

Coppleson M. and Reid B. (1968). Management of cervical carcinoma-in-situ. Lancet 2: 873.

Creagan E.T., Ingle J.N., Green S.J., Ahmann D.L. and Jlang N-S. (1980). Phase II study of tamoxifen in patients with disseminated malignant melanoma. Cancer Treat. Rpt. 64(2-3): 199-201.

Crissman H.A. and Tobey R.A. (1974). Cell cycle analysis in 20 minutes. Science 184: 1297-1298.

Danforth D.N.Jr., Russell N., McBride C.M. (1982). Hormonal status of patients with primary malignant melanoma. A review of 313 cases. South Med. J. 6: 661-664.

Das Gupta T. and Brasfield R. (1964). A clinicopathological study. Cancer 17: 1323-1339.

Defares J.G. and Sweddan I.N. (1964)(eds.) The mathematics of medicine and biology. North Holland Publishing Company, Amsterdam. pp. 1-669.

Denys W.D. (1972a). Studies correlating the growth rate of a tumor and its metastases and providing evidence for tumor-related systemic growth-retarding factors. Cancer Res. 32: 374-379.

Denys W.D. (1972b). A quantitative model for the study of the growth and treatment of a tumour and its metastases with correlation between proliferative state and sensitivity to cyclophosphamide. Cancer Res. 32: 367-373.

De Ridder L.I., Mareel M. and Vakaet L. (1977). Invasion of malignant cells into cultured embryonic substrates. Arch. Geschwulstforsch 97: 7-27.

Detter J.C., Ways P.O., Giblett E.R., Baughan M.A., Hopkinson D.A., Povey S. and Harris M. (1968). Inherited variations in human phosphohexose isomerase. Ann. Hum. Genet. 31: 329-338.

Detre S.I. (1980). Comparison of growth of xenografts in various kinds of immunodeficient mice. In: Immunodeficient Animals for Cancer Research (ed.) S. Sparrow. MRC Lab. Animals Centre Symposium Number 2. The MacMillan Press Ltd., New York.

De Vore D.P., Houchens, D.P., Ovejera A.A., Dill G.S. Jr. and Hutson T.B. (1980). Collagenase inhibitors retarding invasion of a human tumor in nude mice. Exp. Cell Biol. 48(5): 367-373.

Dexter D.L., Matook G.M., Meitner P.A., Bogaars H.A., Jolly G.A., Turner M.D. and Calabresi P. (1982). Establishment and characterization of two human pancreatic cancer cell lines tumorigenic in athymic mice. Cancer Res. 42: 2705-2714.

Domenech R.J., Hoffman J.I.E., Noble M.I.M., Saunders K.B., Henson J.R. and Subijanto S. (1969). Total and regional coronary blood flow measured by radioactive microspheres in conscious and anaesthetized dogs. Circulation Res. 25: 581-596.

Easty D.M. and Easty G.C. (1974). Measurement of the ability of cells to infiltrate normal tissues in vitro. Br. J. Cancer 29: 36-49.

Easty G.C. and Easty D.M. (1984). In vivo and in vitro models of invasion. In: Invasion Experimental and Clinical Implications, (eds.) Mareel M.M. and Calman K.C. Oxford University Press, Oxford, New York, Tokyo pp. 24-62.

Eccles S.A. and Alexander P. (1975). Immunologically-mediated restraint of latent tumour metastasis. Nature 257: 52.

Elder D.E., Dupont Guerry C.B., Heiberger R.M., La Rossa D., Goldman L.I., Clark W.H.Jr., Thompson C.J., Matozzo I. and van Horn M. (1983). Optimal resection margin for cutaneous malignant melanoma. Plast. Reconstr. Surg. 71: 66-72.

Ellerbroek W.C. (1968). Oral contraceptives and malignant melanoma. J. Am. Med. Assoc. 206: 649-650.

Elwood J.M. and Lee J.A. (1974). Trends in mortality from primary tumours of skin in Canada. Can. Med. Assoc. J. 110: 913-915.

Elwood J.M., Lee J.A.H., Walter S.P., Mo T. and Green A.E.S. (1975). Relationship of melanoma and other skin cancer mortality to latitude and ultraviolet radiation in the United States and Canada. Int. J. Epidemiology 3(4): 325-332.

Enterline H.T. and Coman D.R. (1950). The ameboid mortality of human and animal neoplastic cells. Cancer 3: 1033-1039.

Farrant J., Knight S.C., McGann L.E. and O'Brien J. (1974). Optimal recovery of lymphocytes and tissue culture cells following rapid cooling. Nature 249: 452-453.

Festing M.F.W. (1981). The Rowett athymic nude rat. In: Thymusaplastic nude mice and rats in clinical oncology. (eds.) G.B.A. Bastert, H.P. Fortmeyer and H. Schmidt-Matthiessen. Gustav Fischer Verlag Stuttgart. New York. pp. 15-23.

Fex G., Adielsson G. and Mattson W. (1981). Oestrogen-like effects of tamoxifen on the concentration of proteins in plasma. Acta Endocrinol. 97: 109-113.

Fidler I.J. (1970). Metastasis: Quantitative analyses of distribution and fate of tumor emboli labelled with ^{125}I-5-Iodo-2'-deoxyuridine. J. Natl. Cancer Inst. 45: 773-782.

Fidler I.J. (1973a). Selection of successive tumour lines for metastases. Nature New Biol. 242: 148-149.

Fidler I.J. (1973b). The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. Eur. J. Cancer 9: 223-227.

Fidler I.J. (1974a). Inhibition of pulmonary metastasis by I.V. injection of specifically activated macrophages. Cancer Res. 34: 1074-1078.

Fidler I.J. (1974b). Immune stimulation - inhibition of experimental cancer metastases. Cancer Res. 34: 491-498.

Fidler I.J. (1975). Mechanisms of cancer invasion and metastasis. In: Biology of Tumours: Surfaces, Immunology and Comparative Pathology 4. Cancer, a Comprehensive Treatise (ed.) F.F. Becker. Plenum Press, New York pp 101-131.

Fidler I.J. (1976). Patterns of tumour cell arrest and development. In: Fundamental Aspects of Metastasis, (ed.) L. Weiss, North-Holland Publishing Company pp. 275-289.

Fidler I.J. and Nicolson G.L. (1976). Organ selectivity for implantation survival and growth of B16 melanoma variant tumour lines. J. Natl. Cancer Inst. 57: 1199-1202.

Fidler I.J., Gersten D.M. and Riggs C.W. (1977). Relationship of host immune status to tumor cell arrest, distribution and survival in experimental metastasis. Cancer 40: 46-55.

Fidler I.J. and Kripke M.L. (1977). Metastasis results from pre-existing variant cells within a malignant tumour. Science 197: 893-895.

Fidler I.J. (1978). Tumor heterogeneity and the biology of cancer invasion and metastasis. Cancer Res. 38: 2651-2660.

Fidler I.J., Gersten D.M. and Hart J.R. (1978). The biology of cancer invasion and metastasis. Adv. Cancer Res. 28: 149-250.

Fidler I.J., Hart I.R. (1981). The implications of tumor heterogeneity for studies on the biology and therapy of cancer metastasis. Biochim. Biophys. Acta 651: 37-50.

Fidler I.J. (1982). Eradication of metastases by tumoricidal macrophages: therapeutic implications. In: Tumor Invasion and Metastasis (ed.) L.A. Liotta and I.R. Hart. Martinus Nijhoff Publishers, Hague, Boston, London. pp. 29-42.

Fidler I.J. and Hart I.R. (1982). Biological diversity in metastatic neoplasms origins and implications. Science 217: 998-1003.

Fidler I.J., Pollack V.C. and Hanna N. (1984). The use of nude mice for studies of cancer metastasis. In: Immune-deficient Animals. 4th International Workshop on Immune-deficient Animals in Experimental Research, (ed.) B. Sordat Chexbres (1982), Karger, Basel pp. 328-338.

Finney D.J. (1964). Statistical methods in biological assay. 2nd edition. London. Charles Griffin pp 570-571.

Fisher B. and Fisher E.R. (1959). Experimental evidence in support of the dormant tumour cell. Science 130: 918-919.

Fisher B., Soliman O. and Fisher E.R. (1969). Effect of antilymphocyte serum on parameters of tumour growth in a syngeneic tumour-host system. Proc. Soc. Exp. Biol. Med. 131: 16-18.

Fisher B., Soliman O. and Fisher E.R. (1970). Further observations concerning effects of antilymphocyte serum on tumor growth with special reference to allogeneic inhibition. Cancer Res. 30: 2035-2042.

Fisher R.I., Neifeld J.P. and Lippmann M.E. (1976). Oestrogen receptor in human malignant melanoma. Lancet 2: 337-339.

Fisher R.I., Neifeld J.P. and Lippman M.E. (1978). Diethylstilbestrol therapy of surgically non-resectable malignant melanoma. Proc. Am. Assoc. Cancer Res. and A.S.C.O. 19: 339.

Fisher B., Gunduz N. and Saffe E.A. (1983). Influence of the interval between primary tumor removal and chemotherapy on kinetics and growth of metastases. Cancer Res. 43: 1488-1492.

Fodstad O., Aass N. and Pihl A. (1980). Assessment of tumour growth and of response to chemotherapy of human melanomas in athymic nude mice. Br. J. Cancer (suppl.) 41: 146-149.

Fodstad O., Hansen C.T., Cannon G.B., Statham C.H., Lichtenstein G.R. and Boyd M.R. (1984). Lack of correlation between natural killer activity and tumor growth control in nude mice with different immune defects. Cancer Res. 44: 4403-4408.

Fogh J., Fogh J.M. and Orfeo T. (1977). One hundred and thirty seven human tumour cell lines producing tumours in nude mice. J. Natl. Cancer Inst. 59:221-226.

Fogh J. and Giovanella B.C. (1978). The nude mouse in experimental and clinical research. Academic Press, Inc., New York, San Fransisco, London.

Folkman J. (1971). Tumor angiogenesis: therapeutic implications. New Engl. J. Med. 285: 1182-1186.

Folkman J., Merler E., Abernathy C. and Williams G. (1971). Isolation of a tumor factor responsible for angiogenesis. J. Exp. Med. 133: 275-188.

Folkman J. (1974a). Tumor angiogenesis. Adv. Cancer Res. 19: 331-358.

Folkman J. (1974b). Tumor angiogenesis factor. Cancer Res. 34: 2109-2113.

Folkman B.H. (1975). Inhibition of tumor angiogenesis mediated by cartilage. J. Exp. Med. 141: 427-439.

Folkman J. (1975). Tumour angiogenesis. In: Cancer: A Comprehensive Treatise III (ed.) F.F. Becker, Plenum Press, New York, London.

Folkman J. and Tyler K. (1977). Tumour angiogenesis: its possible role in metastasis and invasion. In: Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy. (eds.) D.B. Day, W.P. Laird, S. Myer, P. Stansley, S. Garattini and M.G. Lewis. Raven Press, New York. pp. 95-103.

Foulds J. (1958). The biological characteristics of neoplasia. In: Cancer (ed.) R.W. Raven, Butterworth and Co. Ltd., London. pp. 37.

Freedman V.H., Brown A.L., Klinger H.P. and Shin S. (1976). Mass production of animal cells in nude mice with retention of cell specific markers. Exp. Cell Res. 98: 143-151.

Freedman V.H. and Shin S. (1978). Use of nude mice for studies on the tumorigenicity of animals cells. In: The Nude Mouse in Experimental and Clinical Research (eds.) J. Fogh and B.C. Giovanella. Academic Press, New York. pp. 353-384.

Freshney R.I. (ed.) (1983). Culture of Animal Cells. Alan R. Liss, Inc., New York. pp. 219-221.

Frindel E., Malaise E.R., Alpen E. and Tubiana M. (1967). Kinetics of cell proliferation of an experimental tumour. Cancer Res. 27: 1122-1131.

Frindel E., Malaise E. and Tubiana M. (1968). Cell proliferation kinetics in five human solid tumours. Cancer 22: 611-620.

Gallez G., Henson J.C. and Waelbroek C. (1973). Growth-stimulating effect of nafoxidine on rat mammary tumor after ovariectomy. Eur. J. Cancer 9: 699-700.

Garbisa S., Kniska K., Tryggvason K., Foltz C., Liotta L.A. (1980). Quantitation of basement membrane collagen degradation by living tumor cells in vitro. Cancer Lett. 9: 359-366.

Garcia H., Gasic G. and Baydak T. (1963). Viable tumor cells in blood and lungs of mice with metastazing and non-metastasizing transplantable tumors. Proc. Soc. Exp. Biol. Med. 113: 914-917.

Geder L., Lausch R., O'Neill F. and Rapp F. (1976). Oncogenic transformation of human embryo lung cells by human cytomegalovirus. Science 192: 1134-1137.

Gellin G.A., Kopf A.W. and Garfinkel L. (1969). Malignant melanoma. A controlled study of possibly associated factors. Arch Dermatol. 99: 43-48.

Gerner R.E., Kitamura H. and Moore G.E. (1975). Studies of tumor cell lines derived from patients with malignant melanoma. Oncology 31: 31-43.

Gershman H. (1982). Three-dimensional models for the study of invasion and metastasis. In: Tumor invasion and Metastasis, (eds.) Liotta L.A. and Hart I.R., Developments in Oncology 7, Martinus Nijhoff Publishers pp. 231-250.

Gershwin M.E., Ikedar M., Kawakami T.G. and Owens R.B. (1977). Immunobiology of heterotransplanted human tumours in nude mice. J. Natl. Cancer Inst. 58: 1455-1461.

Gill Grantley P.G., Young N.J., Thompson A., Keightley D.D. and Horsfall D.J. (1984). The effect of tamoxifen on the growth of human malignant melanoma in vitro. Eur. J. Cancer Clin. Oncol. 20(6): 807-815.

Giovanella B.C., Yim S.O., Stehlin J.S., Williams L.J. (1972). Development of invasive tumours in "nude" mouse after injection of cultured human melanoma cells. J. Natl. Cancer Inst. 48: 1531-1533.

Giovanella B.C. and Stehlin J.S. (1973). Heterotransplantation of human malignant tumours in "nude" thymusless mice. I. Breeding and maintenance of "nude" mice. J.N.C.I. 51(2): 615-619.

Giovanella B.C. et al (1974). Influence of the host's sex on the growth of human tumours heterotransplanted in "nude thymusless mice". Proc. Am. Assoc. Cancer Res. 15: 92.

Giovanella B.C. and Stehlin J.S. (1974). Assessment of the malignant potential of cultured cells by injection in nude mice. In: Proceedings of the First Internation Workshop on Nude Mice (eds.) J. Rygaard and C.O. Povlsen. pp 279-284.

Giovanella B.C., Stehlin J.S., Santamaria C., Yim S.O., Morgan A.C., Williams L.J. Jr., Leibovitz A., Fialkow P.J. and Mumford D.M. (1976). Human neoplastic and normal cells in tissue culture. I. Cell lines derived from malignant melanomas and normal melanocytes. J.Natl. Cancer Inst, 56(6): 1131-1142.

Giovannella B.C., Stehlin J.S., Williams L.J., Lee S.S., Shepard R.C. (1978). Heterotransplantation of human cancer into nude mice: a model system for human cancer chemotherapy. Cancer 42: 2269-2281.

Giovanella B.C. and Fogh J. (1978). Present and future trends in investigations with nude mouse as a recipient of human tumor transplants. In: The Nude Mouse in Experimental and Clinical Research (eds.) J. Fogh and B.C. Giovanella. Academic Press, New York pp. 282-290.

Giovanella B.C. (1980). Human tumours as xenografts. Oncology supplement - Scientific Foundation of Oncology (eds.) Sir Thomas Symington and Richard Lawrence Carter. William Heinemann Medical Books, London pp. 73-89.

Giovanella B.C., Stehlin J.S., Shepard R.C. and Williams L.J. (1983). Correlation between response to chemotherapy of human tumors in patients and in nude mice. Cancer 52(7): 1146-1152.

Giovanella B.C., Stehlin J.S. and Coli D. (1984). Human tumors heterotransplanted in nude mice and rats. In: Immune-deficient Animals. 4th International Workshop on Immune-Deficient Animals in Experimental Research, Chexbres 1982, (ed.) B. Sordat, Karger, Basel pp. 76-79.

Goldenberg D.M and Pavia P.A. (1981) Malignant potential of murine stromal cells after transplantation of human tumours into nude mice. Science 212:65-67.

Goldenberg D.M and Pavia P.A. (1982) In vivo horizontal oncogenesis by a human tumour in nude mice. Proc. Natl Acad. Sci. U.S.A.79:2389-2392

Golinger R.C., Gregorio R.M., Fisher E.R. (1977). Tumor cells in venous blood draining mammary carcinomas. Arch. Surg. 112: 707-708.

Gordon H. and Sweets H.H. (1936). A simple method for the silver impregnation of reticulum. Am. J. Pathol. 12: 545-551.

Gorelike E., Segal S., Freedman M. (1978). Growth of a local tumor exerts a specific inhibitory effect on progression of lung metastases. Int. J. Cancer 21: 617-625.

Graham F.L. and van der Eb A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456-467.

Greene H.S.N. and Harvey E.K. (1964). The relationship between the dissemination of tumor cells and the distribution of metastasis. Cancer Res. 24: 799-811.

Griffiths J.D. and Salsbury A.J. (1965). In: Circulating Cancer Cells. (ed.) I.N. Kugelmass. Charles C. Thomas, Springfield,III pp. 74-135.

Griffiths J.D., McKinna J.A., Rowbotham H.D. (1973). Carcinoma of the colon and rectum: circulating malignant cells and 5-year survival. Cancer.31: 226-236.

Gullino P.M., Ediger R.D., Giovanella B., Merchant B., Outzen H.C., Reed N.D. and Wortis H.H. (1976). Guide for the care and use of the nude (thymus deficient) mouse in biomedical research. In: Inst. Lab. Anim. Resources Monograph, Committee on Care and Use of the "nude" mouse. NRC, Natl. Acad. Sci. In: ILAR News, 16, M3.

Hadley J.A. (1952). Case of malignant melanoma in pregnancy. J. Obstet. Gynaecol. Br. Emp. 59: 217-219.

Hansen C.T. (1977). The nude gene in animal models for oncologic research. IN: Proc. Symp. on the use of athymic (nude) mice in cancer research. (eds.) D.P. Houchens and A.A. Ovejera. Gustav Fischer Verlag. Stuttgart. pp. 11-21.

Harper M.J.K., Walpole A.L. (1967). A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J. Reprod. Fertil. 13: 101-119.

Hart I.R. and Fidler I.J. (1978). An in vitro quantitative assay for tumor cell invasion. Cancer Res. 38: 3218-3224.

Hata J-I., Veyama Y., Tamaoki N., Furukawa T., Morita K. (1978). Human neuroblastoma serially transplanted in nude mice and metastases. Cancer Res. 42: 468-473.

Hayman E.G., Engvall E. and Rouslahti E. (1981). Concomitant loss of cell surface fibronectin and laminin from transformed rat kidney cells. J. Cell Biol. 88: 352-357.

Helmrich S.P., Rosenberg L., Kaufman D.W., Miller D.R., Schottenfeld D., Stolley P.D. and Shapiro S. (1984). Lack and elevated risk of malignant melanoma in relation to oral contraceptive use. J.Natl.Cancer Inst. 72(3): 617-620.

Helson L., Das S.K. and Hajdu S.I. (1975). Human neuroblastoma in nude mice. Cancer Res. 35: 2594-2599.

Hersey P. (1983). Expression of Thy-1 antigen on human melanoma cells. Int. J. Cancer.32(1): 21-25.

Hewitt H.B. and Wilson C.A. (1959). A survival curve for mammalian leukaemia cells in vivo. Br. J. Cancer 13: 69.

Hewitt H.B., Chan D. P-S. and Blake E. (1967). Survival curves for clonogenic cells of a murine keratinising squamous carcinoma. Int. J. Radiat. Biol. 12: 535-549.

Hewitt H.B., Blake E. and Porter E.H. (1973). The effect of lethally irradiated cells on the transplantability of murine tumours. Br. J. Cancer 28: 123-134.

Hibbs M.S, Postlethwaite A.E., Mainardi C.L., Seyer J.M. and Kang A.H. (1983) Alterations in collagen production in mixed mononuclear leukocyte-fibroblast cultures.J.Exp. Med. 157:47-59

Hirohashi S., Shimosato Y., Kameya T., Nagai K. and Tsunematsu R. (1977). Hormonal dependency of a serially transplantable human breast cancer (Br-10) in nude mice. Cancer Res. 37: 3184-3189.

Hoal E.G., Wilson E.L. and Dowdle E.B. (1982). Variable effect of retinoids on two pigmenting human melanoma cell lines. Cancer Res. 42: 5191-5195.

Hoal E.G., Wilson E.L. and Dowdle E.B. (1983). The regulation of tissue plasminogen activator activity by human fibroblasts. Cell 34: 273-279.

Hoal-van Helden E.G., Wilson E.L. and Dowdle E.B. (1986). The establishment and characterization of seven melanoma cell lines. Br. J. Cancer, August (in press).

Hoffman J.C. (1973). The influence of photoperiods on reproductive functions in female mammals. Handbook of Physiology (ed.) R.O. Greep. Endocrinology II, Part 1. American Physiological Society, Washington D.C. pp. 57-77.

Hollinshead A., Arlen M., Yonemoto R., Cohen M., Tanner K., Kundin W.D. and Scherrer J. (1982). Pilot studies using melanoma tumor-associated antigens (TAA) in specific-active immunochemotherapy of malignant melanoma. Cancer 49: 1387-1409.

Holly E.A., Weiss N.S. and Liff J.M. (1983). Cutaneous melanoma in relation to exogenous hormones and reproductive factors. J. Natl. Cancer Inst. 70(5): 827-831.

Holt P.G. and Keast D. (1977). Environmentally induced changes in immunological function. Acute and chronic effects of inhalation of tobacco smoke and other atmospheric contaminants in man and experimental animals. Bacteriol. Rev. 41: 205-216.

Houchens D.P. and Ovejera A.A. (1978) (eds). Proceedings of the symposium on the use of athymic (nude) mice in cancer research. Gustav Fischer, New York, Stutgart.

Houghton A.N., Eisinger M., Albino A.P., Cairncross J.G. and Old L.J. (1982). Surface antigens of melanocytes and melanomas: markers of melanocyte differentiation and melanoma subsets. J. Exp. Med. 156: 1755-1766.

Hutterer F. and Singer E.J. (1960) A modified method for hydroxyproline determination. Anal. Chem. 32: 556-558

Jaffe E.A., Mosher D.F. (1978). Synthesis of fibronectin by cultured human endothelial cells. J. Exp. Med. 147: 1779-1791.

Johnson R.O., Bisel H., Andrews N., Wilson W., Rochlin D., Segaloff A., Krementz E., Aust J. and Ansfield F. (1966). Phase I clinical study of 6 alpha-methyl pre-GN-4-ene 3, 11, 20-Trione (NSC-17256). Cancer Chemother. Rep. 50: 671-673.

Jordan V.C., Rowsby L., Dix C.J., Prestwich G. (1978). Dose-related effect of non-steroidal antioestrogen and oestrogens on the measurement of cytoplasmic oestrogen receptors in the rat and mouse uterus. J. Encocrinol. 78: 71-81.

Kameya T., Shimosato Y, Tumvraya M., Ohsawa N. and Nomura T. (1976). Human gastric choriocarcinoma serially transplanted in nude mice. J.Natl. Cancer. Inst. 56: 325-332.

Kanzaki T., Hashimoto K. and Bath D.W. (1979). Heterotransplantation of human malignant melanoma cell lines in athymic nude mice. J. Natl. Cancer Inst. 62(5): 1151-1153.

Karakousis C.P., Lopez R.E., Bhakoo H.S., Rosen R., Moore R. and Carlson M. (1980). Estrogen and progesterone receptors and tamoxifen in malignant melanoma. Cancer Treat. Rep. 64: 819-817.

Kellner B. and Sugar J. (1967). Morphological factors accompanying growth and invasion. In: Endogenous Factors Influencing Host-Tumor Balance, (ed.) R.W. Wissler, T.L. Dao and S. Wood Jr. University of Chicago Press, Chicago. pp. 239-248.

Ketcham A.S., Wexler H. and Mantel N. (1959). The effect of removal of a "primary" tumour on the development of spontaneous metastases. I. Development of a standardized experimental technique. Cancer Res. 19: 940-944.

Ketcham A.S., Kinsey D.L., Wexler H. and Mantel N. (1961). The development of spontaneous metastases after the removal of a primary tumor. II Standardization protocol of five animal tumors. Cancer (Phila.) 14: 875-862.

Kindred B. (1978). Nude mice in immunology. Progress in allergy. 26: 137-238.

Kindred B. and Wechsler W. (1978). Comparison of rat nervous system tumour clones grown in syngeneic rats and mice. In: Proceedings of the symposium on the use of athymic (nude) mice in cancer research. (eds.) D.P. Houchens and A.A. Ovejera, Gustav Fischer, New York. Inc. pp. 145-150.

Kinsey D.L. (1960). An experimental study of preferential metastases. Cancer 13: 674-676.

Kledzik G.S. and Meites J. (1974). Reinitiation of estrous cycles in light-induced constant estrous female rats by drugs. Proc. Soc. Exp. Biol. Med. 146: 989-992.

Kleinerman J. and Liotta L. (1977). Release of tumor cells. In: Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy, (eds.) S.B. Day, W.P. Laird, S. Myer, P. Stansly, S. Garattini and E.T. Lewis. Raven Press, New York. pp 135-143.

Kleinman H.K., Klebe R.J., Martin G.R. (1981). Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol. 88: 473-485.

Kokoschka E.M., Spona J., Bieglmayer C.H. and Schmidt J. (1979). Hormone receptor analysis in malignant melanoma patients. Cancer Treat. Rep. 63: 1200-1284.

Koono J., Ushijima K. and Hayashi H. (1974). Studies on the mechanisms of invasion in cancer III. Purification of a neural protease or rat ascites hepatoma cell associated with production of chemotactic factor for cancer cells. Int. J. Cancer 13: 105-115.

Kozlowski J.M., Fidler I.J., Campbell D., Xu Z-I, Kaighn M.E. and Hart I.R. (1984). Metastasis behaviour of human tumor cell lines growth in the nude mouse. Cancer Res. 44: 3522-3529.

Kramer R.H., Gonzalez R. and Nicolson G.L. (1980). Metastatic tumour cells adhere preferentially to the extra cellular matrix underlying vascular endothelial cells. Int. J. Cancer 26: 639-645.

Krog, H-H. (1976). Identification of inbred strains of mice, mus musculus. I. Genetic control of inbred strains of mice using starch gel electrophoresis. Biochemical Genetics 14(3-4): 319-326.

Laennec R.T.H. (1812). Sur les melanoses. Bulletins de la Faculte de Medecine de Paris 1:2.

Laird A.K. (1964). Dynamics of tumour growth: comparison of growth rates and extrapolation of growth curve to one cell. Br. J. Cancer 9: 278-291.

Laird A.K. (1965). Dynamics of tumour growth: comparison of growth rates and extrapolation of growth curve to one cell. Br. J. Cancer 19: 278-291.

Langer R., Brem H., Falterman R., Klein M. and Folkman J. (1976). Isolation of a cartilage-factor that inhibits tumor neovascularization. Science 193: 70-72.

Lederman J.S., Lew R.A., Koh H.nd Sober A.J. (1985). Influence of estrogen administration on tumor characteristics and survival in women with cutaneous melanoma. J.Natl. Cancer Inst. 74(5): 981-985.

Le Douarin, N. (1982) (ed.) The neural crest. Cambridge University Press, Cambridge/New York. pp. 1-195.

Leighton J. (1951). A sponge matrix method for tissue culture. Formation of organized aggregates of cells in vitro. J. Natl. Cancer Inst. 12: 545-562.

Lerner A.B., Nordlund J.J. and Kirkwood J.M. (1979). Effect of oral contraceptives and pregnancy on melanomas. The New Engl. J. Med. 301(1): 47-48.

Levi J.E. and Lewison E.F. (1952). Malignant melanoma in a patient with ovarian agenesis. Case report of prolonged survival. J. Clin. Endocrin. 12: 901-907.

Lewis M.H., Hill J.T., Leopold J.C. et al. (1982). Guidelines for management of malignant melanoma of the lower limbs. Based on a study of long term behaviour. Clin. Oncol. 8: 341-349.

Liao S.K., Dent P.B. and McCulloch P.B. (1975). Characterization of human malignant melanoma cell lines. I. Morphology and growth characteristics in culture. J. Natl. Cancer Inst. 54(5): 1037-1044.

Lindmo T. and Steen H.B. (1977). Flow cytometric measurement of the polarization of fluorescence from intracellular fluorescein in mammalian cells. Biophys. J. 18: 173-187.

Liotta L.A., Kleinerman J., Saidel G.M. (1974). Quantitative relationships of intravascular tumor cells, tumor vessels and pulmonary metastases following tumor implantation. Cancer Res. 34: 997-1004.

Liotta L.A., Kleinerman J., Catanzaro P., Rynbrandt P. (1977). Degradation of basement membrane by murine tumour cells. J. Natl. Cancer Inst. 58: 1427-1431.

Liotta L.A., Tryggvason K., Garbisa S., Hart J., Foltz C.M., Shafie S. (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284: 67-68.

Liotta L.A., Tryggvason K., Garbisa S., Gehron-Robey P., Abe S. (1981). Partial purification and characterization of a neutral protease which cleaves type IV collagen. Biochemistry 20: 100-104.

Liotta L.A. (1986). Tumor invasion and metastases - role of the extracellular matrix: Rhoads Memorial Award Lecture. Cancer Res. 46: 1-7.

Loor F., Roelants G.E., Kindred B., Mayor K.S. and Hagg L-B. (1976). The differentiation of the T-lymphoid system. The athymic (nude) mouse as a tool. In: Progress in Differentiation Research. (ed.) N. Muller-Berat, Amsterdam: North Holland Publishing Co., pp. 559-566.

Lozzio B.B., Lozzio C.B. and Machado E. (1976a). Human myelogenous (Ph+) leukemia cell line: transplantation into athymic mice. J. Natl. Cancer Inst. 56: 627-629.

Lozzio B.B., Machado E. and Lozzio C.B. (1976b). Hereditary asplenic-athymic mice: transplantation of human myelogenous leukemic cells. J. Exp. Med. 143: 225-231.

Lunscken C. and Strauli P. (1975). Penetration of an ascitic reticulum cell sarcoma of the golden hamster into the body wall and through the diaphragm. Virchows Arch. (Pathol. Anat.) 17: 247-259.

Mackie R.M. and Young D. (1984). Human malignant melanoma. Int. J. Dermatol. 23(7): 433-443.

Martin L. and Middleton E. (1978). Prolonged oestrogenic and mitogenic activity of tamoxifen in the ovariectomized mouse. J. Endocrinol. 78: 125-129.

Masiel A., Buttrick P. and Bitran J. (1981). Tamoxifen in the treatment of malignant melanoma. Cancer Treat. Rep. 65(5-6): 531-532.

McCormick D., Wallace I., Kirk J., Dinsmore S. and Allen I. (1983). The establishment and characterization of a cell line and mouse xenografts from a human malignant melanoma. Brit. J. Exp. Path. 64: 103-115.

McGovern V.J., Shaw H.M., Milton G.W. and McCarthy W.H. (1982). Ulceration and prognosis in cutaneous malignant melanoma. Histopathology 6: 399-407.

McGuire W.L., Carbone P.P., Sears M.E. and Escher G.C. (1975). Estrogen receptors in human breast cancer. An overview, estrogen receptors in human breast cancer. (ed.) W.L. McGuire, P.P. Carbone and E.P. Vollmer. New York, Raven Press, pp. 1-7.

Merenda C., Sordat B., Mach J.P. and Carrel S. (1975). Human endometrial carcinomas serially transplanted in nude mice and established in continuous cell lines. Int. J. Cancer 16: 559-570.

Meyer J.E. and Stolbach L. (1978). Pretreatment radiographic evaluation of patients with malignant melanoma. Cancer 42: 125-126.

Meyskens F.L. Jr. and Voakes J.B. (1980). Tamoxifen in metastatic malignant melanoma. Cancer Treat. Rep. 64: 171-173.

Microbial control of germfree animals - Procedures for Examining category *** and AAAA animals (germ free). Appendix D. In: Microbiological Examination of Laboratory Animals for Purposes of accreditation. Medical Research Council Laboratory Animal Centre, Carshalton, Surrey.

Moore G.E., Lehner D.F., Kikuchi Y. and Less L.A. (1962). Continuous culture of a melanotic cell line from the golden hamster. Science 137: 980-987.

Morton D.L., Eiber F.R. and Holmes E.C. (1974). BCG immunotherapy of malignant melanoma: summary of a 7 year experience. Ann. Surg. 180: 635-643.

Murthy A.S.K. and Russfield A.B. (1970). Endocrine changes in two strains of mice exposed to constant illumination. Endocrinology 86: 914-917.

Nathanson L., Hall T.C. and Farber B. (1967a). Biological aspects of human malignant melanoma. Cancer 20: 650-655.

Nathanson L., Hall T.C., Vawter G.F. and Farber S.(1967b). Melanoma as a medical problem. Arch. Intern. Med. 119: 479-492.

Neifeld J.P. and Lippman M.E. (1980). Steroid hormone receptors and melanoma. The J. Invest. Dermat. 74: 375-381.

Neri A. and Nicolson G.L. (1981). Phenotypic drift of metastatic and cell surface properties of mammary adenocarcinoma cell clones during growth in vitro. Int. J. Cancer 28:731-738.

Nesbit R.A., Woods R.L. and Tattersall M.H.N. and Fox R.M. (1979). Tamoxifen in malignant melanoma. The New Engl. J. Med. 301(22): 1241-1242.

Nichols E.A. and Ruddle F.H. (1973). A review of enzyme polymorphism, linkage and electrophoretic conditions for mouse and somatic cell hybrids in starch cells. The Journal of Histochemistry and Cytochemistry 21(12): 1066-1081.

Nicolson G.L. (1974). The interactions of lectins and animal cell surfaces. Int. Rev. Cytol. 39: 89-190.

Nicolson G.L. and Winkelhake J.L. (1975). Organ specificity of blood-borne tumour metastasis determined by cell adhesion? Nature (Lond.) 255: 230-232.

Nicolson G.L., Winkelhake J.L. and Nussey A.C. (1976). An approach to studying the cellular properties associated with metastasis: some in vitro properties of tumour variants selected in vivo for enhanced metastases. In: Fundamental Aspects of Metasases, (ed.) L. Weiss. North-Holland, Amsterdam pp. 291-303.

Nicolson G.L. and Winkelhake J.L. (1976b). An experimental approach to studying organ specificity of pulmonary tumor metastasis. In: Cell Surfaces and Malignancy, Fogarty International Cancer Proceedings 28, (ed.) P. Mora. U.S. Government Printing Office, Washington D.C.

Nomura T., Oshana N., Tamaori N. and Fujiwara K. (1977) (eds.) Proceedings of the second international workshop on nude mice. Gustav Fischer, New York / Stutgart.

Nowell P.C. (1976). The clonal evolution of tumor cell populations. Science 194(4260): 23-28.

Nowell P.C. (1986). Mechanisms of tumor progression. Cancer Res. 46:2203-2207.

Oettgen H.F., Aoki T., Old L.J., Boyse E.A., de Harven E. and Mills G.M. (1968). Suspension culture of a pigment-producing cell line derived from a human malignant melanoma. J.Natl. Cancer Inst. 41: 827-831.

Ossowski L., Unkeless J.C., Tobia A., Quigley J.P., Rifkin D.B. and Reich E. (1973). An enzymatic function associated with transformation of fibroblasts by oncogenic viruses II. Mammalian fibroblasts cultured transformed by DNA and RNA tumor viruses. J. Exp. Med. 137: 112-126.

Ossowski L. and Reich E. (1980). Experimental model for quantitative study of metastasis. Cancer Res. 40: 2300-2309.

Ossowski L. Reich E. (1983). Changes in malignant phenotype of a human carcinoma conditioned by growth environment. Cell 33(2): 323-333.

Ozaki T., Yoshida K., Ushijima K. and Hayashi H. (1971). Studies on the mechanisms of invasion in cancer. II. In vivo effects of a factor chemotactic for cancer cells. Int. J. Cancer 7; 93-100.

Ozzello L. and Sordat M. (1980). Behaviour of tumours produced by transplantation of human mammary cell lines in athymic nude mice. Eur. J. Cancer 16: 553-559.

Pack G.T. and Scharnagel I.M. (1951). Prognosis of malignant melanoma in pregnant women. Cancer 4: 342-334.

Pantelouris E.M. (1971). Brief communication: observation on the immunobiology of "nude" mice. Immunology 20: 247-252.

Pawelek J., Wong G., Sansone M. and Morowitz J. (1973). Molecular biology of pigment cells. Molecular controls in mammalian pigmentation. Yale J. Biol. Med. 46: 430-443.

Pawelek J. (1979). Melanoma cells. Methods of Enzymology Vol. LVIII: 564-570.

Peters L.J. and Hewitt H.B. (1974). The influence of fibrin formation on the transplantability of murine tumour cells: implications for the mechanisms of the Revesz effect. Brit. J. Cancer 29: 279-291.

Peters R.H. (1983). The ecological implication of body size. Cambridge University Press.

Porter E.H., Hewitt H.B. and Blake E.R. (1973). The transplantation kinetics of tumour cells. Brit. J. Cancer 27: 55-62.

Poste G. (1977). The cell surface and metastases. In: Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy, (ed.) S.B. Day., W.P. Laird., S. Myer., P. Stansly., S. Garattini and E.T. Lewis. Raven Press, New York. pp. 19-47.

Poste G. and Fidler I.J. (1980). The pathogenesis of cancer metastases. Nature (Lond.) 283: 139-145.

Poste G., Doll J., Hart I.R. and Fidler I.J. (1980). In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties. Cancer Res. 40: 1636-1644.

Poste G. (1982). Methods and models for studying tumor invasion. In: Tumor Invasion and Metastasis. (ed.) L.A. Liotta and I.R. Hart. Developments in Oncology 7, Martinus Nijhoff Publishers pp. 147-172.

Potter M., Wax J.S., Anderson A.D., and Nordan R.P. (1985). Inhibition of plasmacytoma development in BALB/c mice by endomethacin. J. Exp. Med. 161:996-1012.

Povlsen C.O. (1977). Heterotransplantation af humane maligne tumorer til nude mus: Copenhagen: F.A.D.L. Publishers, Copenhagen and J. Wiley and Sons, London.

Prehn L.M. and Outzen H.C. (1977). Primary tumour immunity in nude mice. Int. J. Cancer 19: 688-691.

Puck T.T. and Marcus P.I. (1955). A rapid method for viable cell titration and clone production with HeLa cells in tissue culture. The use of X-irradiated cells to provide conditioning factors. Proc. Natl. Acad. Sci. USA 41: 432.

Quastler H. and Sherman F.G. (1959). Cell population kinetics in the intestinal epithelium of the mouse. Exp. Cell Res. 17: 420.

Rampen F.H.J. and Mulder J.H. (1980). Malignant melanoma : an androgen-dependent tumour? Lancet i: 562-565.

Rampen F.H.J. (1982). Malignant melanoma: sex differences in response to chemotherapy. Eur. J. Cancer Clin. Oncol. 18: 107-110.

Rao C.N., Barsky S.H., Terranova V.P. and Liotta L.A. (1983). Isolation of a tumor cell laminin receptor. Biochem. Biophys. Res. Commun. 111: 804-808.

Rapin A.M.C. and Burger M.M. (1974). Tumor cell surfaces: general alterations detected by agglutinins. Adv. Cancer Res. 20: 1-91.

Reid L., Wolf B., Ninayama G., Kaplan N. and Sato G. (1977). Development of transplantable human tumours in nude mice. Proc. Am. Assoc. Cancer Res. 18:161

Reid L.M., Holland J., Jones C., Wolf B., Niwayama G., Williams R., Kaplan N.O. and Sato G. (1978). Some of the variables affecting the success of transplantation of human tumours into the athymic nude mouse. In: Proceedings of the symposium on the use of athymic (nude) mice in cancer research. (eds.) D.P. Houchens and A.A. Ovejera. Gustav Fischer. New York.pp.107-121

Reintgen D.S., McCarty K.M.Jr., Cox E. and Seigler H.F. (1982). Malignant melanoma in black American and white America populations. A comparative review. J.Am. Med. Assoc. 248: 1845.

Révész L. (1956). Effect of tumor cells killed by X-rays upon the growth of admixed viable cells. Nature 178: 1391-1392.

Révész L. (1958). Effect of lethally damaged tumour cells upon the development of admixed viable cells. J. Natl. Cancer Inst. 20: 1157-1186.

Révész L., Littbrand B. and Modig H. (1967). Variation in the response of differnt cells within a tumour to treatment with X-rays or alkylating substances. In Control of Cellular Growth in Adult Organisms. (ed.) H. Teir and T. Rytoma. London and New York, Academic Press p. 374.

Riccardi C., Puccetti P., Santoni A. and Hebberman R.B. (1979). Rapid in vivo assay of mouse natural killer cell activity. J. Natl. Cancer Inst. 63: 1041-1045.

Ricklefs R.E. (1967). A graphical method of fifty equations to growth curves. Ecology 48: 978-983.

Robbins J.C. and Nicolson G.L. (1975). Surfaces of normal and transformed cells. In: Cancer: A Comprehensive Treatise, Vol. IV. (ed.) F.F. Becker, Plenum Press, New York. pp. 1-54.

Roblin R., Chou I-N. and Black P.H. (1975). Proteolytic enzymes and viral transformation. Adv. Cancer Res. 22: 203-252.

Rofstad E.K., Fodstad O. and Lindmo T. (1982). Growth characteristics of human melanoma xenografts. Cell Tissue Kinet. 15: 545-554.

Rogers G.S., Kopf A.W., Rigel D.S. (1983). Effect of anatomical location on prognosis in patients with clinical stage I melanoma. Arch. Dermatol. 119: 644-649.

Romsdahl M.M. and Hus T.C. (1972). Establishment and characterization of human malignant cell lines grown in vitro. In: Pigmentation : Its Genesis and Biologic Control. (ed.) V. Riley. Appleton-Century-Crofts, New York, pp. 461-477.

Rychter Z., Holub M. and Vanecek R. (1978). Topical and quantitative analysis of the thymus region in the nude mouse. Folia Biologica (Praha) Vol. 24: 414-415.

Rygaard J. (1969) Immunobiology of the mouse mutant ((nude)). Acta Path. Microbiol. Scand. 77: 761-762.

Rygaard J. and Povlsen C.O. (1969). Heterotransplantation of a human malignant tumor to the mouse mutant nude. Acta Path. Microbiol. Scand. 77: 758-760.

Rygaard J. (1973). Thymus and self. Immunobiology of the mouse mutant nude. Copenhagen F.A.D.L. Publishers, Copenhagen and J. Wiley and Sons, London.

Rygaard J. (1974). Skin grafts in nude mice. Acta Path. Microbiol. Scand. Section A. 82: 80-112.

Rygaard J. and Povlsen C.O. (1974). The mouse mutant nude does not develop spontaneous tumours. Acta Path. Microbiol. Scand. B. 82: 99-106.

Rygaard J. and Povlsen C.O. (1974)(eds.). Proceedings of First International Workshop on Nude Mice. Gustav. Fischer Verlag Stuttgart-New York.

Rygaard J. and Povlsen C.O. (1976). The nude mouse vs. the hypothesis of immunological surveillance. Transpl. Rev. 28: 43-61.

Rygaard J. (1981). Immunodeficiency in Nude Mice. In: Thymusaplastic Nude Mice and Rat in Clinical Oncology. (eds.) G.B.A. Bastert, H.P. Fortmeyer, H. Schmidt-Matthiesen. Gustav Fischer Verlag-Stuttgart-New York pp. 3-14

Sadoff L., Winkley J. and Tyson S. (1973). Is malignant melanoma an endocrine-dependent tumor? Oncology 27: 224-257.

Salsbury A.J. (1975). The significance of the circulating cancer cell. Cancer Treatm Rev. 2: 55-72.

Sanford K.K., Earle W.R. and Likely G.D. (1948). The growth in vitro of single isolated tissue cells. J. Natl. Cancer Inst. 9: 229-246.

Satyaswaroop P.G., Zaino R.J. and Mortel R. (1984). Estrogen-like effects of tamoxifen on human endometrial carcinoam transplanted into nude mice. Cancer Res. 44: 4006-4010.

Schatten W.E. (1958). An experimental study of post-operative tumour metastases. I. Growth of pulmonary metastases. Following total removal of primary leg tumor. Cancer (Phila.) 11: 455-459.

Schnipper L.E. (1986). Clinical implications of tumour-cell heterogeneity. N. Engl. J. Med. 314:1423-1431.

Schor S.L. (1980). Cell proliferation and migration on collagen substrata in vitro. J. Cell Sci. 41: 159-175.

Shafie S.M. and Grantham F.H. (1981). Role of hormones in the growth and regression of human breast cancer cell (MCF-7) transplanted into athymic nude mice. J.Natl. Cancer Inst. 67(1): 51-56.

Shah J.P. and Goldsmith H.S. (1972). Prognosis of malignant melanoma in relation to clinical presentation. Am. J. Surg. 123: 286-288.

Sharkey F.E. and Fogh J. (1979). Metastasis of human tumours in athymic nude mice. Int. J. Cancer 24: 733-738.

Sharkey F.E. and Fogh J. (1984). Consideration in the use of nude mice for cancer research. Cancer Met. Reviews 3: 341-360.

Shaw H.M., Milton G.W., Farago S. (1978). Endocrine influences on survival from malignant melanoma. Cancer 42: 669-677.

Shaw H.M., McGovern V.J., Milton G.W., Farago G.A. and McCarthy W.H. (1980). Malignant melanoma: influence of site of lesion and age of patient in the female superiority in survival. Cancer 46: 2731-2735.

Shaw H.M. and Milton G.W. (1981). Smoking and the development of metastases from malignant melanoma. Int. J. Cancer 28: 153-156.

Sheridam J.D. (1976). Cell coupling and cell communication during embryogenesis. In: The Cell Surface in Animal Embryogenesis and Development, (ed) G. Poste and G.L. Nicolson. North Holland, Amsterdam. pp. 409-448.

Shirakawa S., Luce J.K., Tannock I. and Frei E. (1970). Cell proliferation in human melanoma. J. Clin. Invest. 49: 1188-1199.

Shimosato Y., Kameya T., Nagai K., Hirohashi S., Koide T., Hayashi H. and Nomura T. (1976). Transplantation of human tumours in nude mice. J. Natl. Cancer Inst. 56(6): 1251-1260.

Shiu M.H., Schottenfeld D., MacLean B. and Fortner J.G. (1976). Adverse effects of pregnancy on melanoma. Cancer 37: 181-187.

Sirbasku D.A. and Benson R.H. (1979). Estrogen-inducible growth factors that may act as mediators (Estromedins) of estrogen-promote tumor cell growth. In: Hormones and Cell Culture, G.Sato and R. Ross (eds.) Cold Spring Harbor Conference on Cell Proliferation, Cold Spring Harbour Laboratory. pp. 477-497.

Skippers H.E. (1983). The forty-year-old mutation therapy of Luria and Delbruck and its pertinence to cancer chemotherapy. Adv. Cancer Res. 40: 331-363.

Sordat B., Fritsche R., Mach J.P., Carrell S., Ozello L. and Cerottini J-C. (1974). Morphological and functional evaluation of human solid tumors serially transplanted in nude mice (abstract). In: Proceedings of the First International Workshop on Nude Mice. (eds.) J. Rygaard and C.O. Povlsen. Stuttgart, Gustav Fischer Verlag. pp 269-278.

Sordat B. and Merenda C. (1977). Invasive growth and dissemination of human solid tumors and malignant cell lines grafted subcutaneously to new born nude mice. (eds.) T. Nomura., N. Ohsawa., N. Tamaoki and K. Fujinara. Tokyo, University Tokyo Press, Stuttgart, Gastav Fischer Verlag. pp. 313-326.

Sordat B.C.M., Ueyama Y. and Fogh J. (1978). Metastasis of tumor xenografts in the nude mice. In: The Nude Mouse in Experimental and Clinical Research. (eds.) B.C. Giovanella and J. Fogh. Academic Press, New York. Vol. 2: 95-147.

Sordat B. and Bogenmann E. (1980). Metastatic behaviour of human colon carcinoma in nude mice. In: Immunodeficient Animals for Cancer Research. (ed.) S. Sparrow. The Macmillan Press Ltd., New York. pp. 145-158.

Sordat B. (1982). Editor of Immune-Deficient Animals. 4th International Workshop on Immune-Deficient Animals in Experimental Research, Chexbres, Karger, New York.

Sousa, de M.A.B., Parrot D.M.V. and Pantelouris E.M. (1969). The lymphoid tissue in mice with congenital aplasia of the thymus. Clin. Exp. Immunol. 4: 637-644.

Spang-Thomsen, M., Nielsen A. and Visfeldt J. (1980). Growth curves of three human malignant tumours transplanted to nude mice. Exp. Cell Biol. 48: 138-154.

Spang-Thomsen, M., Vindeløv L., Christensen I.J., Visfeldt J. and Nielsen A. (1982). Effect of single-dose X-irradiation on growth delay and flow cytometric DNA distribution of a human colonic carcinoma transplanted to nude mice. In: Proceedings of the 3rd International Workshop on Nude Mice. New York, Gustav Fischer Verlag pp. 665.

Spang-Thomsen M. and Vindeløv, L.L. (1984). Proliferation kinetics of a human malignant melanoma serially grown in nude mice. Cell Tissue Kinet. 17: 401-410.

Sparrow S. (1980) editor. Immunodeficient animals for cancer reserach. MRC Laboratory Animal Centre Symposium Number 2. The MacMillan Press Ltd., London.

Sporn M.B. and Todaro G.J. (1980). Autocrine secretion and malignant transformation of cells. The New Engl. J. Med. 303: 878-880.

Sporn M.B. and Roberts A.B. (1985). Autocrine growth factors and cancer. Nature 313: 745-747.

Stark M. and Schlipkoter H.W. (1981). The heterogeneity of human bronchogenic carcinomas in in vitro and in vivo models of the nu/nu mouse and Rnu/Rnu rat. In: Thymusaplastic nude mice and rats in clinical oncology. (Eds.) G.B.A. Bastert, H.P. Fortmeyer and H. Schmidt-Matthiessen. Gustav Fischer Verlag-Stuttgart, New York. pp. 343-349.

Stark M. (1981). Bronchogenic carcinomas in athymic animals. In: Thymusaplastic Nude Mice and Rats in Clinical Oncology. (eds.) G.B.A. Bastert, H.P. Fortmeyer, H. Schmidt-Matthiessen. Gustav Fischer Verlag. Stuttgart, New York. pp. 337-341.

Steel G.G. (1967). Cell loss as a factor in the growth rate of human tumors. Eur. J. Cancer 3: 381-387.

Steel G.G. (1977). The growth kinetics of tumours. Chapter 2 and 4 Oxford Calendar Press, Oxford.

Stevens R.G., Lee J.A.H. and Moolgavkar S.H. (1980). No association between oral contraceptive and malignant melanomas. The New Engl. J. Med. 24: 966-998.

Stewart H. (1955). A case of malignant melanoma and pregnancy. Br. Med. J. 1: 647-649.

Stiles C.D., Desmond W., Chuman L.M., Sato G. and Saier M.H. Jr. (1976a). Growth control of heterologous tissue culture cells in the congenitally athymic nude mouse. Cancer Res. 36: 1353-1360.

Stiles C.D., Desmond W., Chuman L.M., Sato G. and Saier M. Jr. (1976b). Relationship of cell growth behavior in vitro to tumorigenicity in athymic nude mice. Cancer Res. 36: 3300-3305.

Stiles C.D. and Kawahara A.B. (1978). The growth behaviour of virus-transformed cells in nude mice. In: The Nude Mouse in Experimental and Clinical Research, (eds.) B.C. Giovanella and J. Fogh. Academic Press, Inc. New York, San Fransisco, London pp. 385-409.

Stiles C.D., Chuman L.M. and Saier Jr. M.H. (1976c). Enhancement of tumorigenicity in athymic nude mice by coinjection of tumour cells and embryonic fibroblasts. J. Cell Biol. 70: 169a.

Strauch L. (1972). The role of collagenases in tumor invasion. In: Tissue Interactions in Carcinogenesis. (ed). D. Tarin, Academic Press, London pp. 399-434.

Stuttman O. (1977). Two main features of T-cell development: thymus traffic and post-thymic maturation. In: Contemporary Topics in Immunobiology Vol. 7 (ed.) O. Stuttman. Plenum Press, New York and London p. 1-46.

Sylven B. and Bois-Svensson I. (1960). Protein content and enzymatic assays of interstitial fluid from some normal tissues and transplanted mouse tumours. Cancer Res. 20: 831-836.

Talmadge J.E, Wolman S.R., Fidler I.J. (1982). Evidence for the clonal origin of spontaneous metastases. Science 217: 361-363.

Tannock I.F. (1969). A comparison of cell proliferation parameters in solid and ascites Ehrlich tumors. Cancer Res. 29: 1527-1534.

Terenius L. (1971). Structure-activity relationships of anti-oestrogens with regard to interaction with 17-B estradiol in the mouse uterus and vagina. Acta Endocrinol. (Copenhagen) 66: 431-447.

Terranova V.P., Rohrbach D.H. and Martin G.R. (1980). Role of laminin in attachment of PAM 212 (epithelial) cells to basement membrane collagen. Cell 22: 719-728.

Terranova V.P. , Rao C.N., Kalebic T., Margulies I.M.K. and Liotta L.A. (1983). Laminin receptor on human breast carcinoam cells. Proc. Natl. Acad. Sci. U.S.A. 80: 44-451.

Thorgeirsson V.P., Turpeenniemi H.U., Ganen T., Williams J.E., Westin E.H., Heilman C.A., Talmadge J.E. and Liotta L.A. (1985). NIH-3T3 cells transfected with human tumor DNA containing activated Ras oncogens express the metastatic phenotype in nude mice. Mol. Cell Biol. 5: 259-262.

Timpl R., Rohde H., Gehron R.P., Rennard S.I., Foidart J.M. and Martin G.R. (1979). Laminin - a glycoprotein from basement membrane. J. Biol. Chem. 254: 9933-9937.

Tveit K.M., Fodstad O., Johannessen J.V. and Olsness (1980). A human melanoma cell line established from xenograft in athymic mice. Brit. J. Cancer 41: 724-733.

Vladimer G.M., Ballard E., Au W., Lampkin B.C. and Martin L.W. (1978). Heterotransplantation of human neural tumors into nude mice. In: Proceedings of the Symposium on the Use of Athymic (Nude) Mice in Cancer Research, (eds.) D.P. Houchens and A.A. Ovejera. Gustav Fischer Verlag, New York Inc. pp. 151-162.

Wallace A.C. (1965). Effect of delayed addition of irradiated cells to small viable tumor inocula. Cancer Res. 25: 355-357.

Wallace D.C., Exton L.A. and McLeod G.R.C. (1971). Genetic factor in malignant melanoma. Cancer 27: 1262-1266.

Ward J.M., Collins M.J. and Parker J.C. (1977). Naturally occurring mouse hepatitis virus infections in the nude mouse. Lab. Animal Sci. 27: 372-376.

Warenius H.M., Freedman L.S., Bleehen N.M. (1980). The response of human tumour xenograft to chemotherapy: intrinsic variation between tumours and its significance in planning experiments. Br. J. Cancer (suppl. 4): 128-132.

Watson C.S., Medina D. and Clark J.H. (1981). Estrogenic effect of nafoxidine on ovarian-dependent and independent mammary tumor lines in the mouse. Endocrinology 108: 668-672.

Weinstein R.S., Merk F.B. and Alroy J. (1976). The structure and function of intercellular junctions in cancer. Adv. Cancer Res. 23: 23-89.

Weiss L., Glaves D., Waite D. (1974). The influence of host immunity on the arrest of circulating cancer cells in tumor-bearing mice. Int. J. Cancer 18: 850-862.

Weiss L. and Poste G. (1975). The tumor cell periphery. In: Scientific Foundations of Oncology. (ed.) T. Symington and R.L. Carter., Heinemann, London. pp. 25-35.

Weiss L. (1976). Biophysical aspects of the metastatic cascade. In: Fundamental Aspects of Metastasis. (ed.) L. Weiss. North-Holland, Amsterdam. pp. 51-70.

Weiss L., Pamela M., Holmes W. and Holmes J. (1983). Liver-to-lung traffic of cancer cells. Int. J. Cancer 32: 79-83.

Weitberg A.B., Weitzman S.A., Destrempes M., Latt S.A., and Stossel T.P. (1983). Stimulated human phagocytes produce cytogenetic changes in cultured mammalian cells. New England Jour. Med. 308:26-30.

Weitzman S.A., and Stossel T.P. (1981). Mutation cause by human phagocytes. Science (Wash. D C). 212:546-547.

Weitzman S.A., and Stossel T.P. (1982). Effects of radical scavengers and anti-oxidants on phagocyte-induced mutagenesis. J. Immunol. 128:2770-2772.

Weitzman S.A., Weitberg A.B., Clark E.P., and Stossel T.P. (1985). Phagocytes as carcinogens: malignant transformation produced by human neutrophils. Science (Wash. D C). 227:1231-1233.

Welch D.R., Milas L., Tomasovic S.P. Nicolson G.L. (1983). Heterogeneous response and clonal drift of sensitivities of metastatic 13762 NF mammary adenocarcinoma clones to gama radiation in vitro. Cancer Res. 43:6-10.

Wellings S.R., Barishak R. and Siegel B.V. (1960). Cytological studies of human malignant cultures in vitro. Cancer Res. 20: 347-350.

Weston J.A. and Abercrombie M. (1967). Cell mobility in fused homo- and heteronomic tissue fragments. J. Exp. Zool. 164: 317-324.

Wexler H. (1966). Accurate identificationof experimental pulmonary etastasis. J. Natl. Cancer Inst. 36: 641-645.

Wheelock E.F., Goldstein L.T., Weinhold K.J., Carney W.P. and Mark, P.A. (1977). The tumor dormant state. In: Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy. (ed.) D.B. Day, W.P. Laird, S. Myer, P. Stansly, S. Garattini and M.G. Lewis. Raven Press, New York. pp. 105-116.

White L.P. (1959). Studies on melanoma. II. Sex and survival in human melanoma. N. Engl. J. Med. 260: 789-797.

Willis R.A. (1973). The spread of tumours in the human body. 3rd Edition, Butterworths & Co., London.

Wilson E.L. and Dowdle E.B. (1978). Secretion of plasminogen activator by normal reactive and neoplastic human tissues cultured in vitro. Int. J. Cancer 22: 390-399.

Wilson E.L., Becker M.L.B., Hoal E.G. and Dowdle E.B. (1980). Molecular species of plasminogen activators secreted by normal and neoplastic human cells. Cancer Res. 40: 933-938.

Wilson E.L., Gärtner M., Campbell J.A.H. and Dowdle E.B. (1984). Growth and behaviour of human melanomas in nude mice. Effect of fibroblasts. In: Immune-deficient Animals. 4th International Workshop on Immune Deficient Animals in Experimental Research. (ed.) B. Sordat. S. Karger/Basel pp. 357-361.

Winchester R.J., Wang C.Y., Gibofsky A., Kunkel H.G., Lloyd K.O. and Old L. (1978). Expression of IA-like antigens on cultured human malignant melanoma cell lines. Proc. Natl. Acad. Sci. USA 75: 6235-6239.

Wiseman L.L. and Steinberg M.S. (1973). The movement of single cells within solid tissue masses. Exp. Cell Res. 79: 468-471.

Wolf E. (1963). Long-term organotypic cultures of human surgical tumors at the expense of substances elaborated by the mesonephros of chick embryo. Natl. Cancer Inst. Monogr. 11: 180-195.

Woodruff M.F.A. and Dunbar N. (1974). The effect of cell dose and distribution on the development of a transplanted tumour. Eur. J. Cancer 10: 533-537.

Wortis H.H., Nehlsen S. and Owen J.J. (1971). Abnormal development of the thymus in "nude" mice. J. Exp. Med. 134: 681-692.

Wortis H.H. (1974). Immunological studies of nude mice. Contemporary topics in Immunobiology 3: 243-363.

Yamashina K., Miller B.E., and Heppner G.H. (1986). Macrophage - mediated induction of drug - resistant variants in a mouse mammary tumour cell line. Cancer Res. 46:2396-2401.

Yang N.S., Kube D., Park C. and Furmanski P. (1981). Growth of human mammary epithelial cells on collagen cell surfaces. Cancer Res. 41: 4093-4100.

Yarnell M.M. and Schnebli H.P. (1974). Release from density-dependent inhibition of growth in the absence of cell locomotion. J. Cell Sci. 16: 181-188.

Yasumura Y., Tashjian A.H. and Sato G.H. (1966). Establishment of four functional, clonal strains of animal cells in culture. Science 154: 1186-1189.

Zava D.T., Harrington N.Y., McGuire W.L. (1976). Nuclear estradiol receptor in the adult rat uterus: a new exchange assay. Biochem. 15: 4292-4297.

Zeidman I., McCutcheon M. and Coman D.R. (1950). Factors affecting the number of tumor metastases experiments with a transplantable mouse tumor. Cancer Res. 10: 357-359.